UNIVERSIDAD PABLO DE OLAVIDE

DEPARTAMENTO FISIOLOGÍA, ANATOMÍA Y BIOLOGÍA CELULAR

Doctorado en Neurociencias

Tesis Doctoral

NEUROMODULATION FOR MOTOR RECOVERY IN STROKE PATIENTS

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Sevilla, 2017

No te rindas, aún estás a tiempo
de alcanzar y comenzar de nuevo,
aceptar tus sombras,
enterrar tus miedos,
liberar el lastre,
retomar el vuelo.
no te rindas que la vida es eso,
continuar el viaje,
perseguir tus sueños,
destrabar el tiempo,
correr los escombros,
y destapar el cielo.
no te rindas, por favor no cedas,
aunque el frío queme,
aunque el miedo muerda,
aunque el sol se esconda,
y se calle el viento,
aún hay fuego en tu alma
aún hay vida en tus sueños.
porque la vida es tuya y tuyo también el deseo
porque lo has querido y porque te quiero
porque existe el vino y el amor, es cierto.
porque no hay heridas que no cure el tiempo.
abrir las puertas,
quitar los cerrojos,
abandonar las murallas que te protegieron,
vivir la vida y aceptar el reto,
recuperar la risa,
ensayar un canto,
bajar la guardia y extender las manos
desplegar las alas
e intentar de nuevo,
celebrar la vida y retomar los cielos.
no te rindas, por favor no cedas,
aunque el frío queme,
aunque el miedo muerda,
aunque el sol se ponga y se calle el viento,
aún hay fuego en tu alma,
aún hay vida en tus sueños
porque cada día es un comienzo nuevo,
porque esta es la hora y el mejor momento.
porque no estás solo, porque yo te quiero.
Mario Benedetti

A mi padre que me enseñó el amor por la curiosidad matemática y científica y por todo lo que nos decimos y lo que no nos hemos dicho…

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Introduction

Background

Stroke is a major public health concern in industrialized countries and the leading cause of disability worldwide: hence, after a stroke a large number of patients remain impaired, mainly for motor deficits [1].

Motor recovery is typically incomplete [2]. After the episode, 60-70% of stroke survivors can't come back to their usual previous life, in terms of activities of daily living with their paretic hand [3].

Nevertheless, stroke patients usually experience partial spontaneous functional recovery; this phenomenon could be due to the reorganization of remaining neural circuits with the final aim of redeveloping the superior control over the muscles deprived of their normal corticospinal inputs [4]. This reorganization represents the phenomenon of brain plasticity that could be defined as any enduring change in cortical properties. Plasticity is continuously modified by experience and learning and seems to be activated after brain lesions in order to regain a new functional homeostasis [4].

In the last decades, plasticity of human brain has been studied in vivo by means of non-invasive brain stimulation (NIBS) techniques.

NIBS allows not only functional evaluation of corticospinal pathways in clinical setting, specifically by means of single pulse

transcranial magnetic stimulation (TMS) [5], but also a measurement of excitatory and inhibitory phenomena due to activation of different neural elements in the stimulated area of the cortex [6-12].

Functional neuroimaging studies have demonstrated that recovered motor function in the paretic hand of chronic stroke patients relies predominantly on reorganized activity within motor areas of the affected hemisphere (AH) [13,14]. Furthermore, changes in gamma-aminobutyric acid (GABA)-ergic activity in perilesional cortex after stroke seem to have a central role in recovery [15]. Furthermore, animal and in vitro models demonstrated that in the acute phase of a stroke various markers of plasticity are at higher levels both in perilesional territories of affected hemisphere [16,17] and remotely in the contralateral unaffected hemisphere (UH) [18]: it could be reasonable that, during this phase, the effects of neurorehabilitation and neuromodulatory therapy might be maximized.

The most influential model of stroke recovery is based on the inter-hemispheric rivalry or competition hypothesis: the AH becomes doubly disabled, both by its own damage and by the increased hindering output from UH, no longer inhibited by the hypo-functioning AH [2,19]. According to this model, increasing the cortical excitability on the affected side and/or reducing the excitability of the unaffected side can favor recovery.

NIBS can modulate cerebral cortex excitability not invasively and seem to be a promising tool for driving plasticity in damaged brain [20].

The changes induced by NIBS, in form of repetitive transcranial magnetic stimulation (rTMS) or transcranial direct current stimulation (tDCS), are related to long-term changes in synaptic transmission analogue to long-term potentiation (LTP) and long-term depression (LTD) [21]. Several recent studies demonstrated that induction of LTP-like effects (by facilitatory rTMS and tDCS) in the stroke hemisphere and LTD-like effects (by inhibitory rTMS and tDCS) in healthy hemisphere can enhance the effects of motor rehabilitation after stroke [22]. Nonetheless, the effects were limited and variable [23]. On the other hand, it was recently demonstrated that rTMS could improve learning via a different mechanism that involves the phenomenon of the so-called "homeostatic" plasticity. Indeed, it was classically demonstrated that neuronal and synaptic activity has a constant tendency toward a stable physiological condition (homeostasis) along with the different SNC systems. So when a system is perturbed, in the context of either a physiological phenomenon (i.e. learning or development) or a pathological condition (i.e. brain lesions), the system globally tends to reach a new equilibrium: this natural tendency towards a new stable condition is called homeostatic plasticity. In this context, it was elegantly demonstrated that a protocol capable of inducing LTD-like effects strongly facilitates motor learning (that is built up by LTP-like phenomena) while protocols inducing LTP-like effects have a less pronounced and short-lived facilitatory effect on learning [24].

In the context of stroke this would predict that, contrary to usual practice that uses facilitatory protocols on the affected hemisphere, an

inhibitory rTMS protocol (that induces LTD-like effects) over the stroke hemisphere would lead to better relearning in stroke patients through mechanisms of homeostatic metaplasticity.

The impact of stroke-induced motor deficit and its prevalence worldwide together with the great feasibility, relatively cheapness and the great safety of NIBS suggests a need to re-evaluate NIBS as an add-on treatment to physical rehabilitation for post-stroke motor deficit [20].

Research efforts are needed for better studying the neurophysiological mechanism of recovery, and the significance of plastic changes observed after a brain lesion. Moreover, a “re-thinking” of the ways of application of NIBS after stroke is needed so far: systematic studies are warranted to better elucidate the effect of rTMS in relation to different parameters of stimulation and to evaluate new strategies of NIBS application.

Increasing the knowledge in these fields will improve the therapeutical approach with this promising technique for promoting stroke recovery.

Transcranial Magnetic Stimulation

TMS was introduced by Barker and colleagues in 1985 as a non-invasive technique for stimulating motor cortex in humans. A brief high-current pulse through an insulated coil held over the scalp produces a single magnetic stimulus. Since the skull is characterized by little impedance to magnetic fields, magnetic pulse reaches cerebral cortex

inducing electric currents. When the current is induced in the motor cortex, at appropriate amplitude, duration, and direction, it depolarizes cortical neurons evoking a motor response in contralateral limb muscles, mainly hand muscles [25]. Since its introduction, TMS was largely used either in clinical or in research settings, not only to evaluate the functional integrity of the corticospinal tract but also for studying more complex phenomena such as excitatory and inhibitory effects depending on the activation of different neural elements in the stimulated area [26].

Paired pulse protocols are designed to give insight into the nature of the cortical circuitry activated by TMS [26].

Short interval intracortical inhibition (SICI) was the most used paired-pulse protocol and was described for the first time by Kujirai et al. (1993). When a subthreshold conditioning stimulus precedes a later suprathreshold test stimulus by using an interval between stimuli inferior to 5 ms, a clear inhibition of motor response is seen. Since the conditioning stimulus was below active motor threshold (AMT), the authors suggested that the interaction was occurring at a cortical level and that the conditioning stimulus was suppressing the recruitment of descending volleys by the test stimulus [27]. Furthermore, they suggested that SICI was GABA-ergic in origin and this hypothesis was posteriorly confirmed in further more recent studies from other groups [28,29]. Nevertheless, in vivo administration of agonists of GABA-A receptors increases the amount of SICI and also increases the inhibition of later descending I-waves [30].

When two stimuli are given at an intensity at or above active motor threshold, facilitation can be observed between them if the intervals between the shocks is around 1.3, 2.5 and 4.3 ms. This interaction between the stimuli is thought to be due to interaction of I-wave inputs in the periodic bombardment of pyramidal neurons [31].

Moreover, afferent inputs can modify the excitability of the motor cortex with a complex time course. Electrical stimulation, applied to peripheral nerves innervating the hand before a TMS pulse, could induce a very short latency inhibition of MEPs that was called short interval afferent inhibition (SAI) [32]. This inhibition begins about 1 ms after the latency of the N20 component of the sensory evoked potential and lasts for about 7 – 8 ms. Previous studies suggested that the inhibition was cortical in origin and this was confirmed in the recordings of descending volleys [33]. Administration of the muscarinic receptor blocker scopolamine reduced the amount of inhibition in both EMG and descending volley, suggesting that the pathway was under cholinergic control [34]. This data was indirectly confirmed in other studies dealing about patients affected by Alzheimer's disease [35] in which a reduction of SAI was detected: the administration of rivastigmine, a cholinergic drug, could restore this form of inhibition [36].

Repetitive Transcranial magnetic stimulation

By delivering repetitive pulses of TMS (rTMS) it is possible to produce effects on cortical circuits that outlast the duration of the stimulation. Responses to single pulse TMS can be suppressed or facilitated depending on the frequency of the conditioning rTMS train: low frequency rTMS (1 Hz or less) delivered to the primary motor cortex produces a lasting decrease in corticospinal excitability [37]; conversely, high frequency rTMS (frequencies higher than 5 Hz), especially at high intensities of stimulation, leads to facilitatory after-effects on corticospinal circuits [38].

Moreover, different protocols of rTMS were developed: Huang and colleagues described a very rapid method of conditioning the human motor cortex using trains of magnetic stimuli similar to those used in animal experiments to induce long-term depression and long term potentiation, named theta burst stimulation (TBS) [39]. They described different patterns of stimulation with different effects on corticospinal excitability: the continuous theta burst stimulation paradigm (cTBS), (a 40 second train of 3 pulses of 50Hz stimulation repeated every 200 ms for a total of 600 stimuli) produced a marked and long lasting suppression of motor cortex excitability and a significant reduction of glutamatergic-related intracortical facilitation; a second pattern of rTMS, the so-called intermittent theta burst stimulation (iTBS) (10 bursts of high frequency stimulation, 3 pulses at 50 Hz, are applied at 5 Hz every 10s

for a total of 600 pulses) produced a long lasting increase in motor cortex excitability [39].

The mechanisms of the modulation of cortical excitability are unclear, but they might be related to long-term changes in synaptic transmission similar to LTP and LTD equiparable to those seen in the hippocampus after its repeated stimulation [40]. rTMS is able to change and modulate activity beyond the stimulation period, so that it has therapeutic potential in patients with neurological and psychiatric disorders [41].

Safety of rTMS

TMS was introduced more than 20 years ago in clinical neurophysiology and no severe adverse events have been reported. Rodent brains have shown no detectable injury after TMS [42]. Tissue from two adult patients with medically intractable epilepsy who underwent temporal lobe resections 4 weeks after approximately 2000 stimuli, in an rTMS study, showed no changes attributable to rTMS [43]. The most serious documented side effect of rTMS is the induction of epileptic seizures caused by train of high stimulus intensities and frequency [44-46].

rTMS can also induce bursts of electromyographic activity in several muscles contralateral to the stimulated hemisphere showing that trains of rTMS applied to the motor cortex induced a spread of cortical excitability. The spread of excitability depends on the intensity and

frequency of the stimuli and probably constitutes an early epileptogenic effect of rTMS [47].

Guidelines for the safe combination of rTMS frequency, intensity and train length to stimulate the motor cortex of healthy subjects were established. It is unclear whether the established parameters are all safe if used to stimulate the motor cortex of chronic stroke patients who have an “epileptic” risk higher than healthy subjects [48]. Recently it has been reported that rTMS above threshold at 20 and 25 Hz is not safe for patients with chronic stroke [49]. However it should be considered that, till now, no serious side effect or seizure induction has been reported in studies exploring the effect of rTMS in stroke patients and in clinical trials applying rTMS in stroke recovery either when stimulation was delivered on healthy hemisphere or on affected one.

Transcranial direct current stimulation (tDCS)

tDCS involves the application of a low-intensity direct current (DC) (with an intensity of 1–2 mA), through two electrodes situated inside saline-soaked sponges and commonly applied over the scalp. These currents can induce depolarization or hyperpolarization of cortical neurons at a subthreshold level, dependent on polarity used: anodal stimulation depolarizes, while cathodal DCS hyperpolarize the underlying tissue [50]. This subthreshold modulation is achieved through particular electrodes montage on EEG mapped sites, permitting a somewhat focal stimulation of brain cortical areas. Although the precise mechanisms underlying its effects are not still known, there is a robust evidence demonstrating that tDCS is able to induce cortical excitability changes: these changes are present up to 1 h after the end of stimulation, when sufficiently long duration was used [51]. tDCS-induced cortical excitability changes were probed by means TMS in different studies and it was demonstrated that, depending on the polarity and intensity used, tDCS could modulate not only corticospinal (cortical motor output; i.e. MEP amplitude) but also intracortical excitability, such as intracortical inhibition and facilitation (i.e. gabaergic and glutamatergic intracortical networks) [52].

Furthermore, when compared to rTMS, tDCS is a relatively cheap, portable and relatively operator-independent technique: these characteristics permit a broader use of tDCS devices also out of

laboratory settings. Furthermore, the portability of tDCS devices renders the technique available for an “on-line” application, during some motor and rehabilitation tasks. Contemporary neuromodulation, in this case by means of tDCS, could boost the after-effects of physical therapy [4]. Furthermore, scientific evidence demonstrates that tDCS could be safely applied in stroke patients and that could change motor cortex excitability in post-stroke deficit [53].

Main neurophysiological TMS hallmarks in stroke patients

Stroke lesions interfere with the physiological coupling and balancing between the two sides of the brain, releasing unaffected hemisphere and suppressing the excitability of the affected one [20]. In other words, the main electrophysiological abnormality in stroke patients is represented by a contemporary reduced excitability of the affected motor cortex, indexed by an increased RMT and a decreased MEP amplitude, and a trend towards an increase in motor cortex excitability of the healthy hemisphere [54-57]. Furthermore, it was demonstrated that, even in a chronic phase (more than 1 year after the acute event) by performing a single session of either iTBS applied on the affected motor cortex or cTBS on the unaffected hemisphere, it is possible to induce an increase in motor cortex excitability of the affected hemisphere [58]. These data seem to confirm that the function of the affected hemisphere is disturbed both by the lesion, considering the increase of excitability after iTBS, and by the strong inhibitory influence of the unaffected hemisphere. Indeed, it was previously demonstrated that a lateralized focal brain lesion impairs the equilibrium of transcallosal reciprocal inhibition between the two hemisphere, leading to a reduction of trascallosal inhibition from AH towards UH and determining an exacerbate hypoexcitability of motor cortex of lesioned hemisphere with a negative influence on motor recovery [22,23]. Several neurophysiological and neuroimaging experimental findings are in favor of this theory: motor recovery seems to be related to reorganization

within the affected hemisphere, particularly linked to an enhancement of cortical excitability and a reduction of intracortical inhibition within AH. Thus, it could be suggested a possible mechanistic involvement of glutamatergic and GABAergic neurotransmission [59,60]. On the other hand it was demonstrated that TMS measure could provide a sort of prognostic factor for motor recovery: the absence of MEP after TMS of lesioned hemisphere, the absence of iTBS-induced MEP modulation on the affected hemisphere linealrly correlated with a bad prognosis, while a trend toward a not significant correlation was found between an increase of UH excitability and motor recovery [54, 58, 61].

These considerations and data fall again into the concept of post-stroke inter-hemispheric competition model: the more motor cortical excitability difference between affected and unaffected hemisphere, the more the transcallosal inhibition from unaffected to affected hemisphere, the more the motor impairment in the affected hand [20].

Based on this theory, two strategies were mainly used to boost motor recovery after a stroke: increasing the excitability of affected hemisphere and reducing the excitability of the healthy one.

rTMS delivered on affected hemisphere in stroke recovery

It has been reported that rTMS with frequency of stimulation of 5 Hz or more increase the excitability of motor cortex in normal subjects. Also in stroke patients is possible to externally modulate cortical

excitability. A direct evidence of an increase in corticospinal activity induced by rTMS of affected hemisphere in a stroke patient was provided in a patient with a dorsal epidural electrode [62]. This effect seems to be mediated by a selective reduction of the excitability of GABAergic networks in the human motor cortex [60] and decrease the GABA related inhibition facilitating practice-dependent plasticity [63]. These data suggest that rTMS of lesioned hemisphere could facilitate plasticity also by modulating GABA activity in the motor cortex. The first published report about the effect of rTMS for stroke recovery was by Uy et al [64]. They applied very low-frequency rTMS over lower limb motor area of affected hemisphere coupled with electric shocks in stroke patients in an observational not controlled: this protocol is named paired associative stimulation (PAS) that is demonstrated to increase motor cortex excitability when the used interstimulus interval (between electrical and magnetic stimuli) is more than 20 ms. Peripheral and cortical stimulation were applied 30 minutes every weekday for four weeks. Improvement in some neurophysiological and functional measure of affected lower limb was observed. Khedr et al. [65] studied a population of 52 unselected acute stroke patients receiving standard inpatient rehabilitation to improve the overall short-term outcome. They performed a sham-controlled study in which affected hemisphere hand motor area was stimulated at 3 Hz for 10 consecutive days. Real rTMS improved patients' clinical scores (NIH Stroke Scale and Scandinavian Stroke Scale) more than sham stimulation (net change around 35%).

However, no benefit was observed in patients with massive middle cerebral artery infarct.

Kim et al [45] applied 10 Hz rTMS (a facilitatory rTMS protocol) in 15 hemiparetic chronic stroke patients and observed its effects on corticospinal excitability and on motor learning. High-frequency rTMS resulted in a significantly larger increase in the MEP amplitude than the sham rTMS, and the change was positively associated with enhanced motor performance accuracy [66].

In a proof of principle study Talelli and coworkers studied six chronic stroke patients with incomplete recovery of the hand under three conditions: excitatory TBS (iTBS) over the stroke hemisphere, inhibitory TBS (cTBS) over the intact hemisphere and sham stimulation. Only iTBS over AH was able to shorten reaction time in paretic hand and increase the excitability of the lesioned. Inhibitory TBS over the healthy hemisphere suppressed the MEPs evoked in the healthy hands but did not change motor behaviour or the electrophysiology of the paretic hands [67]. Subsequently, in a double-blind placebo control study, developed in collaboration with our group, it was demonstrated that in a population of chronic stroke patients with mild to moderate deficits of upper-limb function, TBS did not augment the gains from a retraining protocol for the upper limb. This study suggests that the concept of a boosting-rehabilitation effect by rTMS for stroke patients is perhaps simplistic. In sum, it was suggested that expectations regarding effect sizes should be reconsidered and, instead of adding treatments together hoping to achieve better results, attention should be focused on

identifying the patients most likely to respond to a particular intervention [68].

rTMS delivered on healthy hemisphere in stroke recovery

Studies in normal subjects showed that low-frequency rTMS is able to increase the excitability of the contralateral motor cortex and reduce the inter-hemispheric inhibition from the stimulated-to-contralateral hemisphere [69] and that could shorten the execution time of a motor task with the ipsilateral hand without affecting performance of the contralateral hand [70].

The first relevant study aimed at evaluating changes in motor function of affected hand induced by rTMS in stroke patients, employed 1 Hz rTMS protocol applied to UH. The authors did not find any significant effect on motor function of the paretic hand [71]. Conversely another research group found that 1 Hz rTMS applied over UH for 10 minutes and with lower intensities in 10 stroke patients induced a significant reduction in simple and choice reaction time and improved performance in affected hand [72]. These results were confirmed in further studies, in which a modulation of duration trans-callosal inhibition was also demonstrated [73] and in which safety and long-lasting effects after 5 consecutive daily sessions of stimulation were shown [74,75].

In general, although safety and feasibility of application of rTMS in both AH and UH is largely confirmed, studies combining standard rehabilitation with rTMS protocols that enhance ipsilesional excitability or

suppress contralesional excitability, based on a non-homeostatic interaction of brain stimulation and motor training, have generated conflicting and variable results [76].

TDCS in motor recovery after stroke

In recent years, the number of studies about the efficacy of tDCS as an add-on treatment has drastically increased. However, although results are generally promising, there is a great variability in the size-effect and in the methodologies used, as such as stimulation montage, intensity, duration and type of outcome measures.

tDCS application to enhance motor recovery after a stroke was mostly based, as like as the application of rTMS, on the concept of a maladaptive interhemispheric disequilibrium [77]. As already stated, the existence of this contralesional inhibitory influence is associated with greater severity of impairment and poorer rehabilitative outcomes [67]. tDCS is thought to be able to directly reverse this imbalance: differently from rTMS, although tDCS could be applied monolaterally upregulating AH (anodal stimulation), or downregulating UH (cathodal stimulation), the advantage of tDCS is represented by the use of both approaches simultaneously (bihemispheric stimulation).

It was hypothesized that anodal tDCS could modulate sodium (Na^+) and calcium (Ca^{2+}) ion channels, NMDA receptors and Gamma-Aminobutyric acid (GABA)-ergic interneurons [51,52]: anodal stimulation

effects are erased when subjects were given Na+ and Ca2+ ion channel blockers and NMDA receptor antagonist block long-term effects [78].

Magnetic resonance spectroscopy also revealed that anodal stimulation could effectively inhibit tonic GABA-ergic activity [79]. Contemporary it was also demonstrated that SICI could be suppressed after a stroke in AH and that motor function recovery has a negative correlation with SICI [80]. Taken together, anodal tDCS, by reducing the GABA-ergic tone in the lesioned cortex, may represent a good strategy to reach an improvement in motor recovery.

UH can be downregulated by means of cathodal stimulation. As like as anodal stimulation, cathodal tDCS depend on NMDA receptors function as well as glutamatergic synapses and interneurons [51,52]. Unlike anodal stimulation though, the administration of Na+ and Ca2+ ion channel blockers had no effect on cathodal after-effects [78]. When anodal and cathodal stimulation are performed contemporary, placing anode on a motor cortex and cathode on the other one, motor performance in the hemisphere stimulated by anode much more than when the same hemisphere was stimulated using unilateral anodal tDCS [81].

In a different study on a group of healthy subjects, it was demonstrated that when a bilateral montage was used (i.e. cathode on a motor cortex and anode on the contralateral one), the effects under the cathode were similar to those induced by monolateral tDCS, while the contralateral effects had a greater size when compared to unilateral cathodal stimulation [82].

The subtle differences in mechanism of action between anodal and cathodal tDCS, together with their gross opposite effects on motor cortex excitability and the possibility of applying them contemporary on lesioned and healthy motor cortex represent a clear advantage in its application for a better motor function recovery after a stroke.

Rationale for the compendium

Non-invasive brain stimulation technique approach needs to be validated yet, keeping particular attention to the expected effect size and the selection of patients that could beneficiate from that.

In stroke patients cohorts, the stimulation of affected hemisphere, that, although the high variability of the available data seems one of the most effective strategy, was criticized mainly for safety concern [83] even tough no relevant adverse event have been reported in the pilot studies in which affected hemisphere was stimulated even in acute phase [48].

For these reasons, at the moment, rTMS for promoting stroke recovery should be used only in research laboratories with experience in rTMS and strictly respecting safety guidelines, even for stimulation of healthy hemisphere [48].

Furthermore the spectrum of stroke patients is characterized by large inter-subjects variability, depending on different parameters as such as age, sex risk factors and, more importantly, lesions' site (cortical vs subcortical) and lesions' volume (small lesions vs large lesions), the lasts accounting for great differences in the recovery prediction. On the other hand, the conflicting results from the NIBS application in promoting motor recovery after a stroke could also depend on the particular neuromodulation protocol applied: within the "family" of inhibitory and facilitatory protocols, each one account for subtle differences in the

mechanisms of action that could affect the ultimate results. Moreover, it was recently demonstrated that genetic background of the patients (see BDNF polymorphisms) could influence the after-effects of neuromodulatory protocols as well as could account (at least in part) for the individual variability in the spontaneous recovery after a stroke in selected cohorts of patients.

Nevertheless, it seems evident that “correction” of inter-hemispheric imbalance, which was the rationale of the majority of the previous studies, failed to obtain univocal and positive results. On the other hand, till now it is not well-known the role of stroke-related UH disinhibition: a recent study showed that application of TMS pulses over the UH hand motor area of well-recovered chronic stroke patients caused a worsening in complex finger movements, suggesting a compensatory and not detrimental role of the increased UH excitability [84].

In line with these findings, few studies demonstrated that the suppression of UH excitability (following the theory of the detrimental inter-hemispheric imbalance) worsens the residual motor abilities in the paretic hand [85]. Furthermore it is well known that a small “ipsilateral” descending motor pathways exists and it is thought to participate mainly in unimanual motor tasks [86]. Consequently, suppressing UH or boosting the excitability of AH could be not the “right” strategy and the opposite approach could be considered, in which the hyperexcitability of UH positively participate in motor recovery: this model is called vicariation/compensatory model.

Moreover, one recent experimental study in a rat model of stroke showed that the artificial reduction of the excitability of UH led to a reduction in motor performance of the paretic forelimb and that the smaller the lesion, the smaller the worsening [87]. With these considerations in mind, Di Pino and colleagues proposed a new model for a tailored treatment in stroke patients, in which competition model should be preferred with small lesions and vicariation/compensatory model with big lesions [88].

Hypothesis and objectives

In the last 6 years my efforts concentrated in the study of the pathophysiology of stroke and motor recovery after a brain lesion. Particularly we built a line of research in which we concentrated in improving NIBS application to boost motor recovery.

In line with these hallmarks, it jumps to attention that NBS is not a “one size fits all” solution for recovery after stroke [89]: this could be true for different aspects, such as inter-individual variability in stroke cohorts, brain lesions variability and the subtle differences between different neuromodulation techniques.

In this compendium I tried to address the following open questions about the application of NIBS in post-stroke motor deficit:

1) The subtle differences between the different NIBS techniques.

2) The significance of plastic changes observed after a brain lesion.

3) The existence (or not) of neurophysiological predictors of NIBS effects in stroke patients.

4) The existence of other NIBS strategy based on new insights of the contribution of the plastic changes induced by the brain lesion.

In order to look for subtle differences between neuromodulation protocols that commonly lead to similar final changes in motor cortex excitability (i.e. increase), we had the great opportunity to study a solid

genetic human model as like as Costello Syndrome (CS), in which a single “gain-of-function” mutation enhances the functionality of the Ras signaling and leads to a detrimental increase in LTP-phenomena. So that, by applying in CS patients two similar neuromodulation protocols capable of inducing LTP, we aimed to disclose any subtle and hidden differences between two similar neuromodulation techniques, by evaluating the influence of a particular mutation in the HRAS system on the effects of TBS and PAS rTMS protocols (paper 1).

Afterwards, in a group of acute stroke patients we explored intracortical excitability of both hemisphere and looked for a possible correlation with motor recovery (paper 2).

Subsequently, in a different study, we tested whether the contemporary application of an excitatory neuromodulation over the affected hemisphere and an inhibitory neuromodulation over the unaffected hemisphere (following the idea of the inter-hemispheric competition/rivalry theory) in the sub-acute phase of a stroke could enhance motor recovery (paper 3).

At last we also tested, in a different group of patients, if, by the application of an inhibitory neuromodulation over the affected hemisphere in chronic stroke patients (by using the rationale of the vicariation/compensation model, in which the increased excitability of the UH is considered compensatory and not detrimental) could improve motor functionality (paper 4).

Methods

Paper 1: Differential Effects of HRAS Mutation on LTP-Like Activity Induced by Different Protocols of Repetitive Transcranial Magnetic Stimulation

We included four patients (mean age: 20.57 ± 4.57 years; age range: 17–27 years) with molecularly confirmed diagnosis of Costello Syndrome. Diagnosis was obtained in relation to clinical characteristics and was confirmed by mutational screening of the HRAS gene [90]. None of the patients was taking drugs acting on CNS or was affected by epilepsy.

A total of 21 age-matched healthy subjects (mean age: 22.1 ± 4.14 years; CS patients vs controls unpaired t-test: $p = 0.43$; age range: 16–34 years) participated in the experiments as a control group.

The present study comprised two independent experiments. Both the experiments consist in three sessions per participant.

Focal TMS of the right hand M1 was performed with a high power Magstim 200 (Magstim Co., Whitland, Dyfed) by using a figure-of-eight coil with external loop diameters of 7 cm held over the right motor cortex at the optimum scalp position to elicit motor responses in the contralateral FDI. Recordings were made from the relaxed first dorsal interosseous muscle (FDI) of the left hand for both groups. The

responses to 20 stimuli obtained at rest at an intensity of 120% RMT were averaged.

iTBS: the protocol was delivered to the right motor cortex over the "hot spot" for the contralateral FDI muscle using a MagPro stimulator (Medtronic A/S Denmark) connected to a figure-of-eight coil (MCF B65). The initial direction of the current induced in the brain was anterior to posterior. The magnetic stimulus had a biphasic waveform. The stimulation intensity was defined in re- lation to AMT evaluated using the MagPro stimulator. An intensity of 80% AMT was used. ITBS protocol consists in the following paradigm: 20 bursts of high frequency stimulation (3 pulses at 50 Hz) are applied at 5 Hz every 10 s for a total of 600 pulses.

Paired associative stimulation (PAS): The intervention consisted of single electrical stimuli delivered to the left ulnar nerve at the wrist at 300% of the sensory perceptual threshold (SPT), followed by TMS at an intensity sufficient to produce an unconditioned response amplitude of approximately 1 mV in the resting FDI. Ninety pairs were delivered at 0.05 Hz over 30 min at an interstimulus interval (ISI) of 25 ms. An ISI of 25 ms was used because this interval had been shown in previous experiments to be effective in increasing cortical excitability [91].

Experimental design: We evaluated motor thresholds and MEP amplitudes in all CS patients and all the healthy subjects in baseline

conditions. In all CS patients and in 16 control subjects we probed the effects of a single session application of iTBS on motor thresholds and MEP amplitudes at two time points, 7 minutes and 30 minutes after the end of iTBS. In all CS patients and in 14 healthy subjects we evaluated the effects of a single session of PAS on motor thresholds and MEP amplitudes at two time points, immediately after and 30 minutes after the end of PAS to replicate the results of our previous study [87]. After TBS and PAS, the amplitude of MEPs was measured using the same stimulus intensity used in baseline conditions even when there was a change in RMT. Twenty sweeps of the data were collected, and the mean peak-to-peak amplitude of MEPs was calculated for each studied groups.

Paper 2: The Level of Cortical Afferent Inhibition in Acute Stroke Correlates With Long-Term Functional Recovery in Humans

Sixteen patients (mean age, 66.8±13.4 years) with first-ever stroke were recruited. Acute-phase evaluation was based on the National Institutes of Health Stroke Scale. Outcome at 6 months was assessed using modified Rankin Scale (mRS). This study was performed according to the Declaration of Helsinki and was approved by the local ethics committee. Patients gave their informed consent before participation.

Patients underwent brain magnetic resonance imaging. Seven patients had a subcortical stroke, whereas 9 patients showed cortical and subcortical involvement. To evaluate whether SAI changes were correlated with structural abnormalities of cholinergic systems, we estimated the damage extent of pathways emanating from nucleus basalis of Meynert: medial pathway, Capsular Lateral pathway, and Perisylvian Lateral pathway [92].

We evaluated active motor threshold and resting motor threshold, amplitude of motor-evoked potentials (MEP), SICI at 2 ms inter-stimulus interval, and SAI at interstimulus intervals from N20 latency plus 2, 3, and 4 ms. We evaluated both affected hemispheres (AH) and unaffected hemispheres (UH).

Because it has been suggested that a change in the slope of input– output curve may influence the amount of cortical inhibition, we also obtained AH input–output curve using increasing stimulus intensities and evaluated whether there was a correlation between slope of input–output curves and amount of AH-SAI [93].

Data obtained in patients were compared with those obtained in 13 healthy subjects (mean age, 70.4±11 years).

Paper 3: Immediate and late modulation of interhemipheric imbalance with bilateral transcranial direct current stimulation in acute stroke

14 patients were recruited for the first experiment: 7 patients underwent real bilateral tDCS and 7 patients underwent sham tDCS. Twenty patients were recruited for the second experiment, 10 patients underwent real bilateral tDCS and 10 patients underwent sham tDCS.

Real/sham tDCS was applied for five continuous days, for 40-min per day. The investigators who applied real/sham tDCS were kept blind to the intervention by using the pre-programmed stimulation mode in the stimulator settings. Participants received tDCS over the primary motor cortex (M1) bilaterally. The excitability-enhancing anode electrode was placed over the primary motor cortex of the AH (C3 or C4 in the 10/20 international EEG system, depending on the lesion side). The excitability-diminishing cathode electrode was placed over the contralateral primary motor cortex of the UH (C3/C4 depending on the lesion side). This montage allows a simultaneous inhibition of the M1 activity in the UH and stimulation in the affected M1. The stimulation parameters were: intensity of 2 mA for 40 min with a current density of 0.057 mA/cm2 (10 s of fade-in and fade-out). We used the same montage as tested by Bolognini et al. and ensured a minimal distance between electrodes of at least 7 cm [94].

Experiment 1: short term clinical effects of tDCS

All patients underwent a standardized protocol of rehabilitation based on physical therapy. An evaluator, blinded to the treatment, assessed the effects of the interventions before the beginning of treatment (baseline assessmentet0) and 1 week after (2 days after the end of the treatmentet1) using the following validated measures sensitive to hand function changes: (i) Action Research Arm Test (ARAT); (ii) 9 Hole Peg Test (9HPT); (iii) Hand- grip strength; (iv) Motor Activity Log Rating Scale (MAL); (v) Score on the National Institutes of Health stroke scale (NIHSS); (vi) modified Rankin Scale score to assess stroke-related disability; (vii) Adverse event monitoring and reporting.

Experiment 2: short- and long-term clinical and electrophysiological effects of tDCS associated with constraint-induced movement therapy

Subjects enrolled in experiment 2 were treated with the association of physical rehabilitation with real/sham bilateral tDCS. All the subjects underwent constraint-induced movement therapy (CIMT), while they were randomized in two groups that received either real or sham bilateral tDCS.

Along 5 days of physical rehabilitation, real/sham tDCS was applied for 40-min during the motor training session, starting 5 min before the beginning of the session.

CIMT was administered for five days by a trained therapist, who was not involved in the pre-post evaluations. The therapy consisted of the association of training tasks designed according to behavioral "shaping" technique while wearing, on the non-paretic hand, a resting splint secured in a sling, which hindered hand and finger activity (SkiL-Care Rigid Palm Padded Mitt; AliMed, Inc, Dedham, Massachusetts). The splint had to be worn for at least 90% of waking hours [94]. During the 5 days of the treatment period, all patients received 1.5 h per day of training of the affected arm in the laboratory [95]. Training tasks were designed to force an intensive use of the paretic extremity, while requiring a progressive improvement of the quality of movement [96]. Nine different shaping tasks were used during this 1.5-h period, which included buttoning a shirt, pouring water, and folding towel.

As part of experiment 2, patients were evaluated at baseline (t0), one week after (t1) and at 3-month follow-up. In these patients, we also explored motor cortex excitability of the AH and UH and the effects of the treatment (Bilateral tDCS and CIMT) on the propensity of the motor cortex of the AH to undergo LTP-like phenomena promoted by using iTBS.

Paper 4: Inhibitory theta burst stimulation of affected hemisphere in chronic stroke: A proof of principle, sham-controlled study.

12 chronic stroke patients gave their written informed consent for the study, which was performed according to the Declaration of Helsinki and approved by the local ethics committee. The National Institute of Health Stroke Scale (NIHSS) and the Barthel Index (BI) were used to evaluate neurological impair- ment and disability at the enrolment. Since this was an exploratory trial in which we aimed to evaluate changes in global hand function, we chose 3 primary outcome measures that evaluate different aspects of that. These were Action Research Arm Test (ARAT; score 0–57), Jebsen-Taylor Test (JTT) and Nine Hole Pegboard Test (NHPT).

We evaluated changes in motor cortex excitability in a subgroup of patients [4 in the real group (mean age: 59.5 ± 11.7 (SD) years) and 4 in the sham group (age: 56.7 ± 16.1; p = 0.5)] of both affected (AH) and unaffected (UH) hemisphere at baseline, T1 and T2.

Continuous Theta Burst Stimulation (cTBS), in which 3 pulses are given at 50 Hz, repeated every 200 ms for a total of 600 pulses at a stimulation intensity of 80% AMT, was applied over hand motor cortex of the affected hemisphere. 6 patients underwent real cTBS and 6 patients underwent sham cTBS: we conducted a double-blind sham controlled study.

Physical therapy included strength training for the wrist, fingers and thumb and grasp and repetitive task practice; the latter aimed mainly at hand function, including, however, proximal elements through functional reach to different areas within the work space.

Papers

Paper 1

Brain Stimulation 9 (2016) 33–38

Contents lists available at ScienceDirect

Brain Stimulation

journal homepage: www.brainstimjrnl.com

Differential Effects of HRAS Mutation on LTP-Like Activity Induced by Different Protocols of Repetitive Transcranial Magnetic Stimulation

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ARTICLE INFO

Article history:
Received 23 April 2015
Received in revised form 21 August 2015
Accepted 24 August 2015
Available online

Keywords:
Plasticity
TMS
HRAS
Costello
iTBS
PAS

ABSTRACT

Background: Costello syndrome (CS) is a rare congenital disorder due to a G12S amino acid substitution in HRAS protoncogene. Previous studies have shown that Paired Associative Stimulation (PAS), a repetitive brain stimulation protocol inducing motor cortex plasticity by coupling peripheral nerve stimulation with brain stimulation, leads to an extremely pronounced motor cortex excitability increase in CS patients. Intermittent Theta Burst Stimulation (iTBS) represents a protocol able to induce motor cortex plasticity by trains of stimuli at 50 Hz. In healthy subjects PAS and iTBS produce similar after-effects in motor cortex excitability. Experimental models showed that HRAS-dependent signalling pathways differently affect LTP induced by different patterns of repetitive synaptic stimulation.
Objective: We aimed to compare iTBS-induced after-effects on motor cortex excitability with those produced by PAS in CS patients and to observe whether HRAS mutation differentially affects two different forms of neuromodulation protocols.
Methods: We evaluated *in vivo* after-effects induced by PAS and iTBS applied over the right motor cortex in 4 CS patients and in 21 healthy age-matched controls.
Results: Our findings confirmed HRAS-dependent extremely pronounced PAS-induced after-effects and showed for the first time that iTBS induces no change in MEP amplitude in CS patients whereas both protocols lead to an increase of about 50% in controls.
Conclusions: CS patients are characterized by an impairment of iTBS-related LTP-like phenomena besides enhanced PAS-induced after-effects, suggesting that HRAS-dependent signalling pathways have a differential influence on PAS- and iTBS-induced plasticity in humans.

Introduction

Costello syndrome (CS; [MIM] 218040) is a rare congenital disorder due to a G12S amino acid substitution in HRAS protoncogene [1] which presents relatively homogeneous clinical phenotype comprising craniofacial anomalies, characteristic cardiac and skeletal problems, developmental delay, mental retardation and certain predisposition to malignancies development [2–4]. Ras genes encode small guanosine triphosphatases (GTPases) that are abundantly expressed in the adult CNS [5]. Several lines of evidence indicate that RAS signalling plays a key role in the mechanisms underlying long-term potentiation (LTP) and learning [6–8].

LTP phenomena have been mostly studied in laboratory settings by using mice brain slice preparation, but the recent introduction of repetitive transcranial magnetic stimulation (rTMS), a non invasive technique able to lastingly modulate the excitability of cortico-spinal and cortico-cortical pathways, has provided a chance for "*in vivo*" evaluation of LTP-like phenomena [9–12].

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1935-861X/
http://dx.doi.org/10.1016/j.brs.2015.08.012

Intermittent theta-burst stimulation (iTBS) is a rTMS protocol, able to induce a lasting increase in motor cortex excitability by using trains of low intensity stimuli and its after-effects, originating at cortical level [13], are dependent on the N-methyl-D-aspartic acid (NMDA) receptors and are linked to motor learning and functional recovery after stroke, suggesting a similarity with those of synaptic LTP [14,15]. It was recently shown that iTBS increases the excitability of cortico-cortical connections of the motor cortex that generate late I-waves in normal subjects as like as another rTMS paradigm called Paired Associative Stimulation (PAS) [16,17]. PAS, obtained by coupling peripheral nerve stimulation with motor cortex stimulation, is based on the Hebbian concept of spike-timing-dependent plasticity [11]. In healthy subjects iTBS and PAS induce similar after-effects on motor cortex excitability [12].

We recently observed a pronounced enhancement of PAS-induced after-effects [18] and the presence of dystonic features in CS [19] in line with previous findings according to which PAS abnormality is involved in the pathophysiology of dystonia [20]. Furthermore, recently it has been demonstrated that PAS enhancement is paralleled by a reduction of iTBS-induced effects in focal hand dystonia (FHD) [21].

Experimental studies have shown that HRAS-dependent signalling pathways in LTP depend on the pattern of synaptic stimulation used [22]. Thus, CS represents a unique model for evaluating "*in vivo*" the relation between HRAS-linked intracellular signalling and LTP-like activity produced by different protocols of non-invasive brain stimulation. The aim of present study was to evaluate PAS- and iTBS-induced brain plasticity in CS patients.

Methods

Ethical approval

The study was performed according to the Declaration of Helsinki and approved by the local Ethics Committee. Informed consent was obtained based on a protocol approved by the institutional review board obtaining the consensus both from patients and their parents after a detailed explanation of the procedures and the aims of the study. The healthy subjects were also informed about the aim of the study and gave their written consent. rTMS was performed according to the recently published guidelines for the use of repetitive transcranial magnetic stimulation in subjects under 18 years [23].

Patients

The study included four patients (mean age: 20.57 ± 4.57 years; age range: 17–27 years) with molecularly confirmed diagnosis. Diagnosis was obtained in relation to clinical characteristics and was confirmed by mutational screening of the *HRAS* gene. None of the patients was taking drugs acting on CNS or was affected by epilepsy. Three patients (patients 1, 2 and 3) were included in a previous paper investigating selectively PAS after effects and were re-evaluated 2 years later.

As in the previous paper by our group [18] Costello patients were evaluated by using the Unified Dystonia Rating Scale (UDRS) Revised score and the Global Dystonia Scale (GDS) in order to quantify dystonic symptoms (Table 1). As described in one previous study from our group, dystonic symptoms in CS go from axial to generalized dystonia, both fluctuating and more evident during walking. Some degree of dystonia was present also at rest [23]. For all the patients, brain and cervical spinal cord magnetic resonance imaging (MRI) were available.

A total of 21 age-matched subjects (mean age: 22.1 ± 4.14 years; CS patients *vs* controls unpaired t-test: $p = 0.43$; age range: 16–34 years) with no known history of neurological disease participated in one or both experiments of the present study to compare their results with those obtained in CS patients. All the studied subjects were right-handed. Nine subjects participated in both experiments.

Table 1
Neurologic, MRI and neuropsychological features in the subjects with Costello syndrome, heterozygous for the c.34G > A missense change (Gly12Ser) in HRAS, included in the study.

	Patient 1	Patient 2	Patient 3	Patient 4
Sex	M	F	F	F
Age (y)	19	17	27	18
Global dystonia Scale	7	6	12	7
Unified Dystonia Rating Scale	5	4	9	6
PAS-induced MEP increase (%)	246*	213*	329*	207*
iTBS-induced MEP increase (%)	3.7**	–20**	3.2**	–3.6**
Epilepsy	–	–	–	–
MRI anomalies	–	Ch	Ch	–
Facial				
"Coarse" face	+	+	+	+
Epicanthus	+	+	+	+
Strabismus	+	+	+	+
Depressed and wide nasal bridge	+	+	+	+
Bulbous tip of nose	–	+	+	+
Fleshy and cocked auricular lobes	+	+	+	+
Low set/posteriorly angulated ears	+	+	+	+
Full cheeks	+	+	+	+
Macrostomy/thick lips	+	+	+	+
Hoarse and deep voice	+	+	+	+
Osteoarticular				
Short neck	+	+	+	+
Ulnar deviation of fingers	+	+	+	+
Interphalangeal laxity of fingers	+	+	+	+
Limited extension of joints	E, H, A	E	E, H, A	H, A
Spine	K	S	HI, S	S

A, achilles tendon; Ch: Chiari 1 malformation; E, elbow; H, hip; HI, hyperlordosis; K, kyphoscoliosis; S, scoliosis; n.e., not evaluated.

* We calculated the upper normal limit of PAS-induced MEP increase in 14 age-matched controls as the mean
MEP increase (%) plus 2 SD, the mean percentage increase in controls was 78% ± 65%, so that an increase greater than 208% was considered outside the normal limits.

** We calculated the lower normal limit of iTBS-induced MEP increase in 17 age-matched controls as the mean
MEP increase (%) less 2 SD, the mean percentage increase in controls was 52% ± 17.8%, so that an increase smaller than 16% was considered outside the normal limits.

The present study comprised two independent experiments. Both the experiments consist in three sessions per participant.

Transcranial magnetic stimulation

Focal TMS of the right hand M1 was performed with a high power Magstim 200 (Magstim Co., Whitland, Dyfed). A figure-of-eight coil with external loop diameters of 9 cm was held over the right motor cortex at the optimum scalp position to elicit motor responses in the contralateral FDI. We decided *a priori to study* right motor cortex both in controls and in CS patients because dystonic symptoms were symmetric. Intensities were expressed as a percentage of the maximum output of the stimulator.

Resting motor threshold (RMT) was defined according to the recommendations of the IFCN Committee [21] as the minimum stimulus intensity that produced a liminal MEP (>50 μV in 50% of 10 trials) with the tested muscle at rest. The active motor threshold (AMT) was defined as the minimum stimulus intensity that produced a liminal MEP (about 200 μV in 50% of 10 trials) during isometric contraction of the tested muscle. Throughout the entire study, both for patients and for healthy subjects, we used an orientation of the stimulating coil over the motor strip with the handle pointing backwards, with the induced current flowing in a posterior–anterior (PA) direction.

MEPs were band pass filtered (bandwidth 3 Hz–3 kHz) (Digitimer D360 amplifiers) and each single trial was recorded on computer for later analysis using a CED 1401 A/D converter (Cambridge Electronic Design, Cambridge, UK) and associated software. Recordings were made from the relaxed first dorsal interosseous muscle (FDI) of the left hand for both groups. The responses to 20 stimuli obtained at rest at an intensity of 120% RMT were averaged. Subjects were given audio-visual feedback of the EMG signal at high gain to assist in maintaining complete relaxation; trials contaminated by EMG activity were discarded.

Intermittent theta burst stimulation

iTBS was delivered to the right motor cortex over the "hot spot" for the contralateral FDI muscle using a MagPro stimulator (Medtronic A/S Denmark) connected to a figure-of-eight coil (MCF B65). The initial direction of the current induced in the brain was anterior to posterior. The magnetic stimulus had a biphasic waveform with a pulse width of about 280 μs and maximum magnetic field strength of 1.5 T. The stimulation intensity was defined in relation to AMT evaluated using the MagPro stimulator. An intensity of 80% AMT was used. ITBS protocol consists in the following paradigm: 20 bursts of high frequency stimulation (3 pulses at 50 Hz) are applied at 5 Hz every 10 s for a total of 600 pulses.

Paired associative stimulation

We used a high power Magstim 200 (Magstim Co., Whitland, Dyfed, UK) connected to a figure-of-eight coil, with external loop diameters of 9 cm held over the right motor cortex at the optimum scalp position to elicit MEPs in the contralateral FDI. The induced monophasic current in the brain flowed in a posterior-to-anterior direction. The intervention consisted of single electrical stimuli delivered to the left ulnar nerve at the wrist at 300% of the sensory perceptual threshold (SPT), followed by TMS at an intensity sufficient to produce an unconditioned response amplitude of approximately 1 mV in the resting FDI. Ninety pairs were delivered at 0.05 Hz over 30 min at an interstimulus interval (ISI) of 25 ms. An ISI of 25 ms was used because this interval had been shown in previous experiments to be effective in increasing cortical excitability [11].

Experimental design

We evaluated motor thresholds and MEP amplitudes in all CS patients and all the healthy subjects in baseline conditions.

In all CS patients and in 16 control subjects (mean age: 23.2 ± 3.8 years; CS patients *vs* controls unpaired t-test: $p = 0.2$) we probed the effects of a single session application of iTBS on motor thresholds and MEP amplitudes at two time points, 7 minutes and 30 minutes after the end of iTBS.

In all CS patients and in 14 healthy subjects (mean age: 23.1 ± 5 years; CS patients *vs* controls unpaired t-test: $p = 0.32$) we evaluated the effects of a single session of PAS on motor thresholds and MEP amplitudes at two time points, immediately after and 30 minutes after the end of PAS to replicate the results of our previous study [18].

After TBS and PAS, the amplitude of MEPs was measured using the same stimulus intensity used in baseline conditions even when there was a change in RMT. Twenty sweeps of the data were collected, and the mean peak-to-peak amplitude of MEPs was calculated for each studied groups.

Seven healthy subjects participated only in iTBS experiment, five healthy subjects only in PAS experiment and nine subjects in both the experiments.

For each CS patient and each subject that participated in both the experiments the intersession interval was at least 1 week to exclude interactions between sessions.

Since some degree of dystonia was present also at rest, we also measured mean rectified EMG activity 100 ms before the stimulus artifact and trials in which EMG activity exceeded ±2.0 SD of the mean resting EMG were excluded from further analysis both for CS patients and controls. A total of 5.6 ± 2.3% trials (both iTBS and PAS sessions) were discarded in CS group and a total of 5.3 ± 1.8% in controls.

Statistics

Since we focused on any difference between iTBS and PAS-induced after-effects upon baseline motor cortex excitability in CS patients, baseline MEP amplitude, RMT and AMT and their changes after each rTMS protocol were compared in Costello patients. Nevertheless we aimed to disclose any difference in the after-effects of each rTMS protocol (iTBS and PAS) between CS patients and the group of age-matched controls.

To compare motor thresholds between the two groups (Costello patients *vs* healthy controls) and after each rTMS protocol we used a four-way factorial ANOVA with TIME (t0, t1, t2), PARAMETER (RMT, AMT) and STIMULATION (iTBS, PAS) as within-subject factors and GROUP (CS patients and controls) as between-subjects factor. To compare MEP amplitudes we used a separated three-way factorial ANOVA with TIME (T0, T1, T2) and STIMULATION (iTBS, PAS) as within-subject factor and GROUP (Costello and controls) as between-subjects factor. When significant main effects or interactions were found, post hoc t-tests with correction for multiple comparisons were performed. The level of significance was set at $P < 0.05$.

Before entering ANOVA, since we studied only 4 CS patients and this number is too small to assume normality, all the raw data were tested for normality by using Shapiro–Wilk test and for sphericity by using Mauchly's sphericity test and, furthermore, we decided to normalize the spread of MEP amplitudes dividing them by the grand mean of the baseline mean values of each group. Using this method, the baseline mean is always equal to 1 but with the same variance as that of the raw data.

To obtain a measure of reproducibility of the PAS after-effects in this rare condition, since 3 out of 4 CS patients included in the present paper were enrolled in a previous paper by our group dealing with PAS after-effects in Costello disease, we compared, by means of 3 different unpaired t-tests, MEP amplitude at the 3 studied time points for the patients that participated in both studies.

To assess a possible correlation between dystonia and the degree of MEP changes in CS patients, we performed Pearson's Chi-squared correlation test for both PAS effects and iTBS effects (MEP changes) *vs* GDS scores.

Results

None of the patients and controls experienced any adverse event during and after the application of rTMS protocols. No significant difference was found in *baseline* cortical excitability parameters between patients and controls. ANOVA for motor thresholds showed no significant TIME × STIMULATION × GROUP interactions ($F_{1,32} = 0.11$; $p = 0.74$).

Details about the mean values of RMT, AMT, and MEP amplitude in baseline condition are reported in Table 2.

ANOVA for baseline-normalized MEPs revealed a significant TIME × STIMULATION × GROUP interaction ($F_{1,16} = 19.372$; $P < 0.001$). Post-hoc unpaired t-test showed no statistical difference for baseline MEPs between CS patients and controls and between the two control groups [CS (iTBS) *vs* controls (iTBS) $p = 0.99$; CS (iTBS) *vs*

Table 2
Mean baseline values (±SD) of motor thresholds and motor evoked potential (MEP) amplitudes in each group (CS patients and controls) for each experimental session (PAS and iTBS).

Groups	RMT (% MSO)	AMT (% MSO)	MEP (mV)
CS *(iTBS session)*	47.7 ± 5.2	35.2 ± 4.8	0.67 ± 0.27
CS *(PAS session)*	46.2 ± 8.6	33.5 ± 5.5	0.6 ± 0.23
Controls *(iTBS session)*	43 ± 10.6	33.9 ± 8.2	0.65 ± 0.25
Controls *(PAS session)*	48.2 ± 11	35.9 ±7.8	0.55 ± 0.27

controls (PAS) p = 0.6; CS (iTBS) *vs* CS (PAS) p = 0.27; CS (PAS) *vs* controls (PAS) p = 0.78; CS (PAS) *vs* controls (iTBS) p = 0.71; controls (iTBS) *vs* controls (PAS) p = 0.34] (see Table 2 for details).

Remarkably, in CS patients mean baseline-normalized MEP amplitude was increased by about 250% immediately after PAS and by approximately 320% when evaluated at 30 minutes after the end of PAS, whereas after iTBS we observed no significant change of MEP amplitude at the two time-points [CS/PAS (baseline *vs* T1 *vs* T2), CS/iTBS (baseline *vs* T1 *vs* T2): 1 ± 0.39 *vs* 3.4 ± 1.5 (p = 0.02) *vs* 4.2 ± 1.8 (p = 0.01); 1 ± 0.4 *vs* 0.45 ± 0.39 (p = 0.87) *vs* 0.93 ± 0.38 (p = 0.8)]. In healthy subjects we observed an increase in baseline-normalized MEP amplitude by about 73% at T1 and by about 39% at T2 after PAS [(T1 *vs* baseline) *vs* (T2 *vs* baseline): (1 ± 0.49 *vs* 1.73 ± 0.87) p < 0.001 *vs* (1 ± 0.49 *vs* 1.39 ± 0.68) p < 0.001] and an increase by about 51% at T1 and by about 40% at T2 after iTBS [(T1 *vs* baseline) *vs* (T2 *vs* baseline): (1.51 ± 0.62 *vs* 1 ± 0.4) p < 0.001 *vs* (1.4 ± 0.52 *vs* 1 ± 0.4) p < 0.001]: post hoc unpaired t-test for comparison of the effects of the two stimulation protocols in healthy group did not reveal any significance, that means that iTBS and PAS produce the same facilitatory effect in baseline-normalized MEP amplitude of controls. (Fig. 1)

When CS were compared to controls, iTBS induced a significant increase in baseline-normalized MEP amplitude in controls, but not in CS patients and conversely we confirmed that PAS induced a significantly more pronounced increase of MEP amplitude in CS when compared to control group (Fig. 1).

We found no correlation between PAS after effects/iTBS after-effects and dystonia severity score (GDS): PAS: R = 0.92 (T1) 0.48 (T2); iTBS: R = 0.71 (T1), 0.84 (T2) [cut-off value for a monodirectional hypothesis 2.84].

The comparison of PAS effects in MEP amplitude changes in the 3 patients that participated in both studies did not reveal any significant difference (T0: p = 0.74; T1: 0.93; T2: 0.95) [baseline MEP amplitude (2 years ago *vs* present study) (mV): 0.67 ± 0.32 *vs* 0.58 ± 0.28 mV; T1 MEP amplitude: 2.2 ±1.08 *vs* 2.1 ± 1.09 mV; T2 MEP amplitude: 2.3 ± 1.09 *vs* 2.25 ± 1.13 mV, thus indicating a good reproducibility of the PAS after-effects in CS.

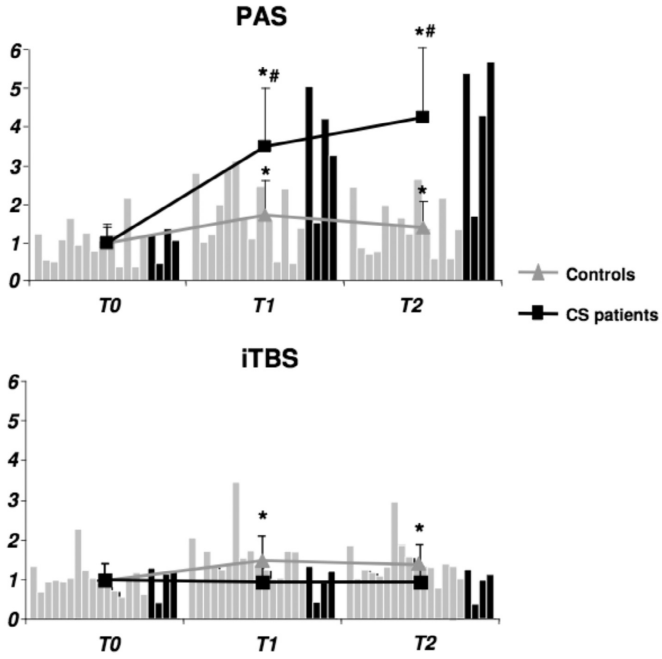

Figure 1. The bar chart illustrates the values of motor evoked potentials (MEPs), each normalized to baseline grand mean value of each group, recorded at rest in baseline conditions, soon after and 30 minutes after the end of PAS and iTBS (in two distinct panels) in CS patients and controls; grey bars represent values for each control subject, black bars represent values for each CS patient to show the variability of MEP amplitudes for each group. The overwritten linear plot schematically shows the time course of the mean after-effects of PAS in CS patients and controls. Error bars represent standard deviations. *: Statistical significance when values were compared *vs* baseline values of the same group (TIME effect); #: Statistical significance when the effects of PAS were compared between CS patients and controls at the same time points.

Discussion

In present study we confirm our previous findings about the effects of facilitatory PAS in CS with a pronounced enhancement of LTP-like activity when compared with healthy subjects [18]: mean MEP amplitude was increased by about 250% immediately after PAS and by approximately 320% when evaluated at 30 minutes after the end of PAS. We also show for the first time that iTBS did not induce any LTP-like activity in Costello patients. In contrast with CS patients, the effects of the two protocols were similar in controls: both iTBS and PAS led to an increase of about 50–70% of MEP amplitude, comparable to data reported in previous studies [11,12,24]. Moreover, no differences between patients and controls were found in baseline TMS parameters. Thus, the main finding of our study is that PAS and iTBS induced differential after-effects in CS patients in contrast with healthy age-matched controls.

Several hypotheses might explain present findings.

Differential after-effects of PAS and iTBS as hallmark of dystonia

A recent paper has shown that FHD patients present an increase in PAS-induced plasticity and a lack of iTBS-induced one when compared to healthy controls [21]. The authors suggested that iTBS-induced LTP-like plasticity was reduced or absent owing to abnormalities in intrinsic M1 circuits, whereas PAS-induced LTP-like plasticity was considered dependent on an altered sensorimotor integration [21]. Thus, since CS patients present mild to moderate dystonic symptoms [19] it could be possible that the absence of iTBS-induced plasticity and the presence of enhanced PAS-induced after-effects could be considered a further neurophysiologic hallmark of dystonia. Although present data are in line with those by Belvisi et al., further suggesting a link between dystonia and the different behaviours of two facilitatory rTMS protocols, it should be considered that CS patients represent a special and unique patients' cohort. Indeed, unlike dystonic patients in which also a reduction in intracortical inhibition was shown [25,26], CS patients are characterized by a normal level of intracortical inhibition [18]. Moreover, the increase in MEP amplitude after PAS in our patients is more pronounced than that observed in focal hand dystonia patients. These differences between CS and dystonic patients suggest that even though it is reasonable to hypothesize that excessive neuroplasticity may play a mechanistic role for the development of dystonia in CS, in analogy with patients with task specific hand dystonia [27], more complex dysfunctional synaptic changes might be present in CS. Moreover, we still do not know whether an abnormal processing of sensory inputs is involved in the pathophysiology of dystonia in CS as reported in focal dystonia [28,29]. Indeed, we only know that somatosensory evoked potentials are normal in CS but for instance sensory discrimination and sensorimotor integration have never been evaluated.

Differential activation of synaptic activity by PAS and iTBS

We suggested in one of our previous papers that dystonia in CS could be due to an altered signalling in HRAS pathways with interferences in synaptic activity of basal ganglia networks [19]. Furthermore, some differences in the neuromodulatory mechanisms of PAS and iTBS were already demonstrated in Parkinson's Disease (PD) patients in which L-dopa was able to restore abnormal plasticity induced by PAS [30–32] but not the abnormal response to iTBS [33]. Also, several lines of experimental evidence support the hypothesis that different stimulation protocols could activate different pathways inducing LTP. Grover and colleagues found that, although continuous stimulation and burst stimulation are both effective in LTP induction, these procedures show a different capability in inducing EPSPs, suggesting a differential effect of the two protocols on NMDA receptors with a different degree of participation of L-type calcium channels [34]. Also, studies in humans have shown that even though different protocols of rTMS produce similar changes in MEP amplitude, the physiological bases of these effects might be different with the involvement of different cortical circuits [35].

Differential role of HRAS in the after-effects induced by different stimulation protocols

Recent evidence in transgenic synRas mice demonstrated that GTPase encoded by Ras genes exerts significant morphoregulatory effects on the dendritic phenotype as well as on the structural and functional synaptic connectivity [36,37]: the mutation responsible for CS upregulates signal flow through this GTPase. After their activation, NMDA receptors stimulate Ras proteins that trigger mitogen-activated protein kinase (MAPK) pathway that, in its turn, activates the final effectors that are critical for LTP induction: selective MAPK inhibitors block synaptic LTP [38]. Although several studies conducted on mice brain slices support the role of HRAS in LTP induction [39,40], it is still unclear whether HRAS suppresses [6,7,41,42] or enhances LTP [8]. Furthermore, it was recently demonstrated that PAS-induced motor cortex plasticity in patients with Neurofibromatosis type 1 (NF1) and Noonan syndrome (NS), belonging together with LEOPARD syndrome and Cardiofaciocutaneous syndrome to a class of genetic syndromes linked to perturbation of function through the Ras pathway [43], was reduced [44,45]. On the other hand in NF-1 mouse models, a defect in CNS dopamine was demonstrated [46] and, contemporary, it was shown that L-DOPA administration restored defective PAS-induced plasticity in PD patients [30]. Hence, since no dopamine defect was demonstrated in CS, it could be argued that dopamine defect in NF-1 could have influenced the results and could be one of the reason for which our results go in an opposite direction. On the other hand, it was shown that carrying a mutated neurofibromin with gain of function mutation in the RAS-MAPK pathway leads to a decrease rather than increase of LTP [40,41]. Hence, the discrepancy between our results about PAS and iTBS-induced plasticity in CS and results about plasticity in Noonan Syndrome and NF-1 could be due, in our opinion, to the different sites of mutation in the different pathologies. For example neurofibromin is the altered multidomain Ras-activating protein in NF-1, Ras is also stimulated by membrane growth factor tyrosine kinase receptors acting through Sos and ShP2 (targets of mutation in Noonan syndrome) [47]. Moreover, due to the complexity of LTP phenomena that involve multiple electrophysiological components of neurons, it was shown that, in NF1 mice brain slices, theta burst stimulation (TBS) effects were reduced while high frequency stimulation (HFS) effects were normal [48]. Furthermore, although LTP induced by high-frequency stimulation (100 Hz) is enhanced in HRAS knock out mice [6], it was shown that low-frequency (5 Hz) stimulation-induced LTP is normal suggesting that the involvement of HRAS-dependent signalling pathways in LTP may be highly dependent on the pattern of synaptic stimulation [8,22]. Based on these assumption it could be argued that HRAS-dependent perturbations of intracellular signalling could affect some key factors in the capability of the different stimulation protocols to induce plasticity, probably by influencing the properties of the post-synaptic neuron [22,41,42].

After considering the neurophysiologic differences between CS patients and FHD patients, the central role of HRAS in pathophysiology of CS and the experimental evidence of an involvement of HRAS in LTP highly dependent on the pattern of synaptic stimulation used, although the non invasive nature of rTMS studies at the system level makes unlikely a strict correspondence between mechanisms underlying *in vivo* LTP-like plasticity and those underlying

experimental LTP [49], our data suggest that HRAS-dependent signalling pathways could differently affect *in vivo* LTP-like phenomena induced by iTBS and PAS. However, despite we studied approximately 1% of the world CS population [50], our study presents the main limitation of the small number of included CS patients and further studies are needed to further elucidate the role of HRAS signalling in synaptic plasticity.

Conclusions

The presence of differential after-effects induced by PAS and iTBS in this cohort of CS could represent not only a complex neurophysiologic marker of dystonia, but could also support the hypothesis of subtle differences in the mechanisms of action of PAS and iTBS in humans and, at last, suggest their different dependence on HRAS signalling pathways.

Acknowledgements

We wish to thank the patients and families who participated in the study. This study was supported in part by the Associazione Italiana Sindromi di Costello e CFC (to G.Z.) and the ERA-Net for research programmes on rare diseases 2009 (to M.T.).

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Paper 2

The Level of Cortical Afferent Inhibition in Acute Stroke Correlates With Long-Term Functional Recovery in Humans

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Background and Purpose—Using transcranial magnetic stimulation, we investigated short-interval intracortical inhibition and short-latency afferent inhibition in acute ischemic stroke.

Methods—We evaluated short-interval intracortical inhibition and short-latency afferent inhibition in the affected hemisphere and unaffected hemisphere in 16 patients and correlated electrophysiological parameters with outcome at 6 months.

Results—Affected hemisphere short-latency afferent inhibition was significantly reduced in patients, and short-latency afferent inhibition level correlated with functional outcome.

Conclusions—Reduced afferent inhibition in acute stroke correlates with long-term recovery. **(*Stroke*. 2012;43:250-252.)**

Key Words: GABA ■ transcranial magnetic stimulation

Changes in gamma-aminobutyric acid (GABA)-ergic activity in perilesional cortex after stroke have a central role in recovery.[1] Inhibitory circuits of human cerebral cortex can be evaluated using paired–pulse transcranial magnetic stimulation, short-interval intracortical inhibition (SICI), or by coupling peripheral nerve stimulation with transcranial magnetic stimulation in short-latency afferent inhibition (SAI).[2] Both inhibitory phenomena are mediated by inhibitory interneurons that use $GABA_A$ receptors, but different receptor subtypes are involved in SICI and SAI.[2]

We investigated SICI and SAI in acute stroke and evaluated the correlation between the level of cortical inhibition and functional outcome at 6 months.

Methods and Patients

Sixteen patients (mean age, 66.8±13.4 years) with first-ever stroke were recruited. Acute-phase evaluation was based on the National Institutes of Health Stroke Scale. Outcome at 6 months was assessed using modified Rankin Scale (mRS). This study was performed according to the Declaration of Helsinki and was approved by the local ethics committee. Patients gave their informed consent before participation.

Patients underwent brain magnetic resonance imaging. Seven patients had a subcortical stroke, whereas 9 patients showed cortical and subcortical involvement. To evaluate whether SAI changes were correlated with structural abnormalities of cholinergic systems, we estimated the damage extent of pathways emanating from nucleus basalis of Meynert: medial pathway, Capsular Lateral pathway, and Perisylvian Lateral pathway.[3] For further details, see Supplemental Methods and Supplemental Figure I (http://stroke.ahajournals.org).

Magnetic Stimulation

We evaluated active motor threshold and resting motor threshold, amplitude of motor-evoked potentials (MEP), SICI at 2 ms interstimulus interval, and SAI at interstimulus intervals from N20 latency plus 2, 3, and 4 ms. We evaluated both affected hemispheres (AH) and unaffected hemispheres (UH).

Because it has been suggested that a change in the slope of input–output curve may influence the amount of cortical inhibition,[4] we also obtained AH input–output curve using increasing stimulus intensities and evaluated whether there was a correlation between slope of input–output curves and amount of AH-SAI.

Data obtained in patients were compared with those obtained in 13 healthy subjects (mean age, 70.4±11 years).

Statistical Analysis

Comparison between AH and UH was performed by means of paired *t*-test, after checking frequency distributions and, eventually, transformed raw values, to achieve a better fit to gaussianity and a reduction of biasing effects of outliers (such as for MEP values). Comparisons of stroke patients versus healthy subjects were performed by means of *t*-test for independent samples.

Associations between electrophysiological findings and clinical outcome (mRS at 6 months) were assessed by means of nonparametric Spearman's rho. The potential effect of the lesion site on mRS was assessed with Mann-Whitney *U* test. Electrophysiological mea-

Received July 1, 2011; accepted July 21, 2011.

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The online-only Data Supplement is available at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.111.631085/-/DC1.

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Stroke **is available at http://stroke.ahajournals.org** **DOI: 10.1161/STROKEAHA.111.631085**

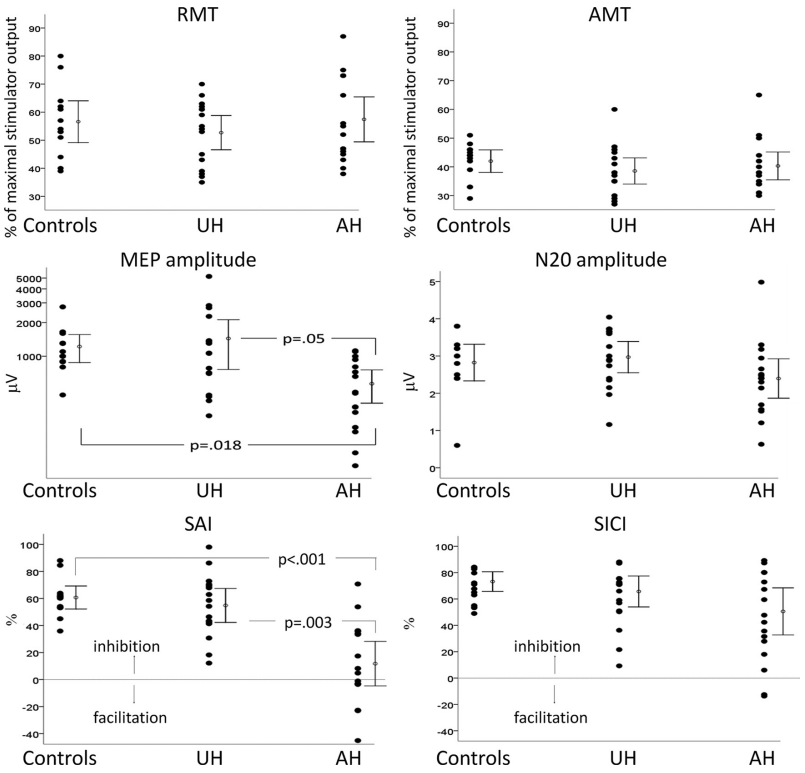

Figure. Electrophysiological findings in patients and controls. Control subjects' and patients' (UH and AH) individual values and 95% CIs of resting motor threshold (RMT) and active motor threshold (AMT), motor evoked potential (MEP) amplitude (*t* test applied after appropriate log-transformation), N20 amplitude, short-latency afferent inhibition (SAI), and short-latency intracortical inhibition (SICI).

sures associated with clinical outcome were further investigated through partial correlation analysis. More specifically, the correlation between AH-SAI and mRS-6-months was controlled for baseline National Institutes of Health Stroke Scale with the following formula:

Here, the left term indicates partial correlation between SAI and mRS, controlling for National Institutes of Health Stroke Scale, and the right term comprises the usual bivariate nonparametric correlations. This measure, after the appropriate transformation, follows a t-distribution with (n−3) degrees of freedom.

Correlation between AH-SAI and the recruitment slope (indexed by the linear increase of MEP amplitude with respect to stimulation increase) was evaluated with Spearman's rho. Because stroke-induced functional changes in cortical excitability may be influenced by stroke location and distribution,[5] we evaluated the effect of lesion site (subcortical or cortical–subcortical) on SAI using Mann-Whitney *U* test.

Nonparametric Spearman's ρ was used to correlate AH-SAI with percentages of lesional voxels in cholinergic pathways.

Significance levels were adjusted according to Bonferroni procedure to control the risk of α-inflation.

Results

Results are summarized in Figure. AH-SAI and AH-MEP amplitude were lower than corresponding UH and control values.

No evidence of association between electrophysiological parameters and stroke severity in the acute phase was found (consistently $P>0.05$). Looking at correlations with clinical status at 6 months, the only significant associations were found with AH-SAI (Table).

When the effect of AH-SAI on mRS was adjusted for the confounding effect of baseline clinical status (National Institutes of Health Stroke Scale at T0), the nonparametric partial correlation remained significant (rho=0.66; $P=0.016$), suggesting its relevance even equalizing for baseline clinical status.

There was no correlation between AH-SAI and either slope of the input–output curve (Spearman's rho=0.12; $P=0.676$) or site of the lesion (Mann-Whitney *U*, $P=0.958$). Also, there was no correlation between site of the lesion and recovery at 6 months (Mann-Whitney *U*, $P=0.99$).

Involvement of cholinergic pathways was limited (Supplemental Table I), and there was no correlation between AH-SAI and either percentage of lesional voxels of medial pathway ($\rho=-0.39$; $P=0.52$), lateral cholinergic pathways

Table. Bivariate Correlation Between Electrophysiological Findings and Clinical Recovery (modified Rankin Scale Score at 6 months)

	Spearman's ρ	Corrected P Value
RMT, UH	−0.04	>0.90
RMT, AH	−0.07	>0.90
AMT, UH	0.37	>0.90
AMT, AH	0.24	>0.90
MEP amplitude, UH	0.29	>0.90
MEP amplitude, AH	0.13	>0.90
SAI, UH	0.29	>0.90
SAI, AH	0.68	0.03
SICI, UH	0.54	0.3
SICI, AH	0.10	>0.90

RMT indicates resting motor threshold; UH, unaffected hemisphere; AH, affected hemisphere; AMT, active motor threshold; MEP, motor-evoked potential; SAI, short-latency afferent inhibition; SICI, short-interval intracortical inhibition.

($\rho=-0.47$; $P=0.264$), and lateral perforant pathway ($\rho=-0.43$; $P=0.376$) or percentage of lesional voxels in the 3 pathways considered together (Spearman's $\rho=-0.5$; $P=0.17$).

Discussion

We report for the first time a suppression of afferent inhibition in acute stroke. AH-SAI level was correlated with recovery at 6 months.

SAI is produced by afferent inputs, and central cholinergic pathways are involved in SAI[2]; thus, a lesion of these circuits might explain its reduction. However, the absence of consistent sensory deficits and/or abnormalities of N20 wave of somatosensory evoked potentials, the limited involvement of cholinergic pathways, and the absence of any correlation between involvement of cholinergic pathways and level of SAI, make this hypothesis unlikely.

We speculate that SAI suppression might be produced by functional changes in central inhibitory circuits.[2] Because SAI is probably mediated by the α5-subunit,[2] we suggest that its suppression might be related to a reduction of activity related to this subunit. Interestingly, a recent experimental study showed that pharmacological antagonization of α5-subunit activity promotes functional recovery after stroke.[1] Long-term potentiation can be induced in motor cortex by stimulation of sensory cortex,[6] and it has been proposed that long-term potentiation produced by sensory inputs might promote cortical reorganization after a lesion.[7] Thus, it can be speculated that reduced SAI level could enhance sensory stimuli-related long-term potentiation phenomena in the motor cortex with a positive effect on relearning related recovery.

Disclosures

None.

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Paper 3

Brain Stimulation 7 (2014) 841–848

Contents lists available at ScienceDirect

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Immediate and Late Modulation of Interhemipheric Imbalance With Bilateral Transcranial Direct Current Stimulation in Acute Stroke

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ARTICLE INFO

Article history:
Received 14 April 2014
Received in revised form
3 September 2014
Accepted 1 October 2014
Available online 28 October 2014

Keywords:
Transcranial direct current stimulation
Transcranial magnetic stimulation
Acute stroke
Recovery

ABSTRACT

Background: Significant changes in neurophysiological and clinical outcomes in chronic stroke had been reported after tDCS; but there is a paucity of data in acute stroke.
Objective: We aimed to evaluate whether a tDCS-induced modulation of primary motor cortex excitability in patients with acute stroke enhances motor recovery associated with rehabilitation and induces differential neuroplasticity.
Methods: We conducted two experiments in acute stroke patients. In experiment 1 (14 patients), we tested the immediate effects of bilateral tDCS alone as compared to sham tDCS on recovery. Experiment 2 (20 patients) was designed to assess effects of bilateral tDCS delivered together with constraint-induced movement therapy (CIMT). In this experiment, we included a longer follow-up (3 months) and measured, in addition to the same clinical outcomes of experiment 1, changes of motor cortex excitability and the amount of promoted LTP-like activity.
Results: Despite the expected improvement at 1 week, none of the clinical measures showed any different modulation in dependence of *CIMT* and *tDCS*. On the neurophysiological assessments, on the other hand, the *Real_tDCS* group, compared to *Sham_tDCS* group, showed a reduction of inter-hemispheric imbalance when considering the differences of motor evoked potential between both 3-month and 1 week follow up ($P = 0.007$) and three month and baseline ($P = 0.015$).
Conclusions: Despite the lack of additional clinical changes, real bilateral tDCS, together with CIMT, significantly reduces inter-hemispheric imbalance between affected and unaffected hemispheres. These findings may shed light on plasticity changes in acute stroke and its potential impact in chronic phases.

Introduction

In recent years, several small studies have reported positive effects of non-invasive cortical stimulation, in the form of repetitive transcranial magnetic stimulation (rTMS) or transcranial direct current stimulation (tDCS), to enhance recovery in patients with acute or subacute stroke [1–3]. However, it is still unknown whether these interventions will be useful in the clinical setting. Neuromodulation techniques can induce long-lasting changes in the excitability of the synapses in motor cortical areas in a manner which might be biologically similar to the long-term potentiation/depression (LTP/LTD) phenomena described at cellular level [4]. LTP/LTD are important for learning and memory and are likely involved in reacquisition of skill after stroke.

Considering that, in the acute phase of stroke, animal and in vitro models have showed that various markers of plasticity are shown to be at higher levels both in perilesional territories of affected hemisphere (AH) [5,6] and remotely in the contralateral unaffected hemisphere (UH) [7], it is reasonable that, during this phase, the effects of neuromodulatory therapy might be maximized.

One of the most influential models of stroke recovery is based on the inter-hemispheric rivalry or competition hypothesis: the

Disclosures: authors have no disclosures.

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http://dx.doi.org/10.1016/j.brs.2014.10.001
1935-861X/

AH becomes doubly disabled, both by its own damage and by the increased hindering output from UH, no longer inhibited by the hypo-functioning AH [8,9]. Although other possible explanations may account for the imbalance of excitability between the two hemispheres (i.e. vicariation of the AH), the inter-hemispheric competition has been exploited so far as rationale by most of the neuromodulatory interventions aimed at improving stroke motor recovery [41]. According to this model, recovery can be favored by increasing the cortical excitability on the affected side and/or reducing the excitability of the unaffected side.

It may then be the case that the bilateral application of tDCS over the motor cortices, with anodal tDCS over the AH and cathodal tDCS over the UH, results in a useful strategy to induce an additive effect compared to unilateral stimulation [10–12].

We designed two experiments to assess whether tDCS-induced bihemispheric modulation in acute stroke patients (tested within 48–96 h of stroke) would modify neuroplasticity and clinical outcomes. In the preliminary small double-blind, randomized, placebo controlled experiment (experiment 1) we evaluated the short-term effects of bilateral tDCS alone on recovery. Since this experiment was negative, we designed another experiment as to assess further plasticity and long-term effects by combining tDCS with constraint-induced movement therapy. Our initial hypothesis was that active tDCS would induce larger motor gains and enhanced neuroplasticity as compared to sham tDCS.

Given the two main techniques of non-invasive brain stimulation, we chose tDCS. Although these two techniques seem to have a final common effect on neuroplasticity, their effects are mediated by different mechanisms of action. A recent experimental study has shown that direct current stimulation applied to rat brain slices has a direct effect on the amount of LTP that can be induced by repetitive stimulation [13]. This is increased by anodal and reduced by cathodal DCS. In analogy with these experimental data, it can be speculated that in the intact human brain, tDCS, even though via subthreshold modulation, can enhance the propensity of the cortex to undergo LTP-like plasticity after rTMS [14,15]. This latter approach means that tDCS exerts his effect especially when coupled with other interventions, thus it might be useful to promote relearning of skills when associated with motor rehabilitation, given also that tDCS is feasible simultaneously with behavioral therapies [16]. Therefore, by combining non-invasive brain stimulation and motor training/learning it should be possible to increase the modulatory effects on the motor neural network and thus increase clinical gains.

We conducted a detailed neurophysiological and clinical assessment. In fact, given the possibility that the assessment of the clinical outcome would not be sensitive enough to all the aspects of the effects induced by tDCS, we evaluated also changes in motor cortex excitability of both hemispheres.

Along this line, we recently showed that the level of LTP-like activity promoted by intermittent theta burst stimulation (iTBS), a robust form of rTMS, delivered to the affected hemisphere (AH) during the acute phase of human stroke correlates with long-term functional recovery [17]. Similarly, suppressive rTMS of the unaffected hemisphere (UH) resulted in the reduction of the AH motor threshold that correlated with recovery [18]. Thus, the neuroplasticity response to neuromodulatory protocols in the acute phase of stroke over both hemispheres is a parameter that might be useful as a surrogate marker of recovery. Accordingly, in this experiment, we also evaluated whether five consecutive days of bilateral tDCS could increase the propensity of the hemispheres to undergo LTP-like plasticity. We used iTBS, which is able to promote LTP-like activity lasting up to 1 h [19], to investigate how tDCS changes iTBS-promoted LTP-like activity.

Materials and methods

Patients

Patients with a history of first ischemic cerebral infarct admitted to the Stroke Unit were enrolled in the study. *Inclusion criteria* were: (1) Age 18–90; (2) Clinical first ever ischemic cerebrovascular accident – confirmed by MRI; (3) Acute phase of stroke (treatment was started 48–96 h after the stroke onset).

Exclusion criteria: (1) Pre-stroke disability; (2) Any substantial decrease in alertness, language reception, or attention that might interfere with understanding instructions for motor testing; (3) Excessive pain in any joint of the paretic limb; (4) Contraindications to single-pulse TMS, such as metal head implants; (5) Advanced liver, kidney, cardiac, or pulmonary disease; (6) Coexistent neurological or psychiatric disease (including epilepsy) as to decrease number of confounders; (7) History of significant alcohol or drug abuse; (8) Use of neuropsychotropic drugs, such as antidepressants or benzodiazepines. The study was approved by the local Ethics Committee. For each experiment, patients were randomized to real or sham tDCS treatment through a block randomization stratification approach (Table 1). This approach ensured that both groups had similar motor impairment.

Fourteen patients were recruited for the first experiment, 7 patients underwent real bilateral tDCS and 7 patients underwent sham tDCS.

Twenty patients were recruited for the second experiment, 10 patients underwent real bilateral tDCS and 10 patients underwent sham tDCS.

Details of demographic and clinical features of each patient are presented in Table 2.

Transcranial direct current stimulation

Direct current was transferred by a saline-soaked pair of surface sponge electrodes (35 cm^2) and delivered by a specially developed, battery-driven, constant current electrical stimulator (Eldith DC-Stimulator, Germany). Real/sham tDCS was applied for five continuous days, for 40-min per day. The investigators who applied real/sham tDCS were kept blind to the intervention by using the pre-programmed stimulation mode in the stimulator settings.

Real tDCS

Participants received tDCS over the primary motor cortex (M1) bilaterally. The excitability-enhancing anode electrode

Table 1
Demographic findings.

	Experiment 1					
	tDCS_sham (7 subjects)		*tDCS_real* (7 subjects)			
	Mean	SER	Mean	SER	Test value (t)	P value
Age	71.71	5.254	66.43	5.956	−0.666	0.518
Onset_Days	2.71	0.421	2.57	0.812	−0.156	0.878
NIHSS admission	7.00	1.345	7.29	2.008	0.118	0.908
mRS admission	3.57	1.134	3.57	0.202	0.000	1.000
Weak side (R/L)	3R/4L		3R/4L			
	Experiment 2					
	tDCS_sham (10 subjects)		*tDCS_real* (10 subjects)			
	Mean	SER	Mean	SER	Test value (t)	P value
Age	68.80	3.681	60.80	5.101	−1.272	0.220
Onset_Days	3.40	0.452	3.10	0.567	−0.414	0.684
NIHSS admission	5.90	0.657	5.80	0.940	−0.087	0.932
mRS admission	3.80	0.200	3.30	0.260	−1.523	0.145
Weak side (R/L)	6R/4L		2R/8L			

Table 2
Demographic and clinical features of patients randomized in the two groups.

		Patient no.	Gender	Age	Days after stroke	Thrombolysis	Paretic side	NIHSS	mRS	Stroke lesion	Vascular territory	TOAST classification
Experiment 1	real tDCS	1	F	79	3	No	R	10	4	Sc	MCA	LAA
		2	M	37	1	No	L	3	3	C/Sc	MCA	CE
		3	M	70	1	No	R	5	4	Sc	MCA	LAA
		4	F	82	1	No	L	17	4	C/Sc	MCA	LAA
		5	M	70	4	No	R	9	4	C/Sc	IC	LAA
		6	M	54	4	No	L	6	3	C/Sc	IC	LAA
		7	M	73	1	No	L	1	3	Sc	PCA	CE
	sham tDCS	8	M	71	2	Yes	L	4	4	C/Sc	MCA	CE
		9	M	80	4	Yes	L	2	1	C/Sc	MCA	LAA
		10	F	77	2	No	L	8	4	C/Sc	ACA/MCA	CE
		11	M	80	3	No	R	6	4	C/Sc	MCA	CE
		12	M	78	2	No	R	9	4	Sc	MCA	LAA
		13	F	41	3	No	R	13	4	C/Sc	IC	OE
		14	F	75	2	Yes	L	7	4	Sc	MCA	LAA
Experiment 2	real tDCS	1	F	51	4	No	L	9	4	Sc	MCA	LAA
		2	M	28	4	No	R	4	2	Sc	MCA	UE
		3	M	68	7	No	L	6	3	C/Sc	ACA/MCA	LAA
		4	F	53	3	Yes	L	7	4	C/Sc	MCA	LAA
		5	F	74	3	No	L	2	3	Sc	MCA	SVO
		6	M	83	2	No	L	3	2	C/Sc	IC	LAA
		7	M	78	4	No	R	6	4	C/Sc	ACA/MCA	LAA
		8	M	56	1	Yes	L	12	4	C/Sc	MCA	LAA
		9	M	53	1	No	L	5	4	C/Sc	MCA	LAA
		10	F	64	2	No	L	4	3	Sc	MCA	CE
	sham tDCS	11	M	53	1	Yes	L	6	4	Sc	MCA	LAA
		12	F	66	4	No	L	5	4	C/Sc	MCA/PCA	CE
		13	M	69	2	Yes	L	7	4	C/Sc	MCA	LAA
		14	M	77	4	No	R	6	4	C/Sc	MCA	CE
		15	F	77	4	No	L	2	2	Sc	MCA	LAA
		16	M	54	4	No	R	7	4	C/Sc	ACA	CE
		17	M	63	4	No	R	10	4	Sc	VB	LAA
		18	M	83	2	No	R	4	4	C/Sc	MCA	LAA
		19	F	86	3	Yes	R	6	4	Sc	MCA	CE
		20	M	60	3	No	R	6	4	C/Sc	IC	LAA

tDCS, transcranial direct current stimulation; M, male; F, female; R, right; L, left; NIHSS, NIH Stroke Scale; mRS, modified Rankin Scale; C, cortical; Sc, subcortical; ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; IC, internal carotid artery; VB, vertebrobasilar; TOAST, Trial of Org 10172 in Acute Stroke Treatment; LAA, large artery atherosclerosis; CE, cardioembolism; SVO, small vessel occlusion – lacunar stroke; OE, other determined etiology; UE, undetermined etiology.

(saline-soaked sponge electrode – 35 cm^2) was placed over the primary motor cortex of the AH (C3 or C4 (10/20 international EEG system) depending on the lesion side). The excitability-diminishing cathode electrode was placed over the contralateral primary motor cortex of the UH (C3/C4 depending on the lesion side). This montage allows a simultaneous inhibition of the M1 activity in the UH and stimulation in the affected M1. The stimulation parameters were: intensity of 2 mA for 40 min with a current density of 0.057 mA/cm^2 (10 s of fade-in and fade-out). We used the same montage as tested by Bolognini et al. and ensured a minimal distance between electrodes of at least 7 cm [20].

Sham tDCS

The same montage and stimulation parameters were employed for sham stimulation, however, current was only applied for 30 s to induce the slight tingling sensation that some subjects report experiencing during tDCS stimulation. This method of sham stimulation has been shown to be reliable [21].

Furthermore, for both real and sham stimulation current intensity was gradually increased (at the beginning of the session) during 10 s and decreased (at the end of the session) also during 10 s to diminish its perception.

Experiment 1: short term clinical effects of tDCS

All patients underwent a standardized protocol of rehabilitation based on physical therapy (Fig. 1 Top Panel). An evaluator, blinded to the treatment, assessed the effects of the interventions before the beginning of treatment (baseline assessment–t0) and 1 week after (2 days after the end of the treatment–t1) using the following validated measures sensitive to hand function changes: (i) Action Research Arm Test (ARAT); (ii) 9 Hole Peg Test (9HPT); (iii) Handgrip strength [22]; (iv) Motor Activity Log Rating Scale (MAL) [23]; (v) Score on the National Institutes of Health stroke scale (NIHSS) [24,25]; (vi) modified Rankin Scale score to assess stroke-related disability; (vii) Adverse event monitoring and reporting.

Experiment 2: short- and long-term clinical and electrophysiological effects of tDCS associated with constraint-induced movement therapy

Subjects enrolled in experiment 2 were treated with the association of physical rehabilitation with real/sham bilateral tDCS. All the subjects underwent constraint-induced movement therapy (CIMT), while they were randomized in two groups that received either real or sham bilateral tDCS (Fig. 1 Bottom Panel).

Along 5 days of physical rehabilitation, real/sham tDCS was applied for 40-min during the motor training session, starting 5 min before the beginning of the session.

CIMT was administered for five days by a trained therapist, who was not involved in the pre-post evaluations. The therapy consisted of the association of training tasks designed according to behavioral "shaping" technique while wearing, on the non-paretic hand, a resting splint secured in a sling, which hindered hand and finger activity (SkiL-Care Rigid Palm Padded Mitt; AliMed, Inc, Dedham, Massachusetts). The splint had to be worn for at least 90% of waking

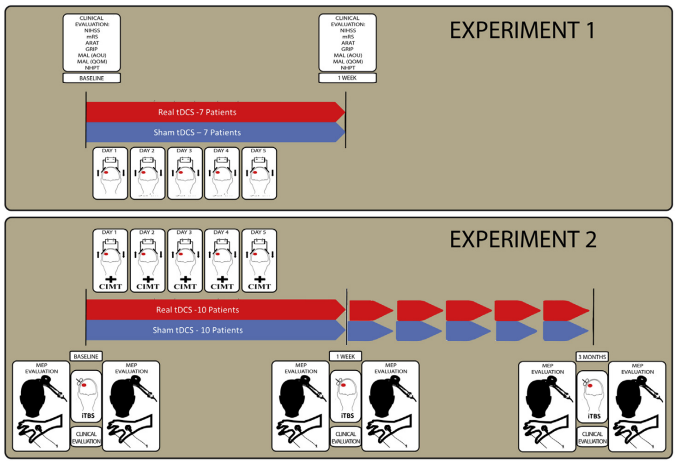

Figure 1. Experimental design. *Top panel*: Experiment 1 is a small double-blind, randomised, placebo controlled experiment to evaluate the short-term effects of bilateral tDCS on recovery. Patients, recruited and treated 48–96 h after the stroke onset, underwent real/sham tDCS (2 mA, current density 0.057 mA/cm^2), applied for five continuous days, for 40-min per day. The effect of the stimulation was assessed through clinical scales (NIHSS = National Institutes of Health stroke scale, mRS = modified Rankin Scale, ARAT = Action Research Arm Test, GRIP = Handgrip strength, MAL (AOU) = Motor Activity Log Rating Scale – Amount of Use, MAL (QOM) = Motor Activity Log Rating Scale – Quality of Movement, NHPT = 9 Hole Peg Test) administered before the beginning of treatment (baseline assessment – t0) and 1 week after (2 days after the end of the treatment – t1). *Bottom panel*: Subjects of experiment 2 underwent constraint-induced movement therapy (CIMT), while they were randomized in two groups that received either real or sham bilateral tDCS (2 mA, current density 0.057 mA/cm^2). Along 5 days of physical rehabilitation, real/sham tDCS was applied for 40-min/day during the motor training session, starting 5 min before the beginning of the session. *Patients*. In these patients, the same clinical scales of experiment 1, AH and UH motor cortex excitability and the effects of the treatment on the propensity of the motor cortex of the AH to undergo LTP-like phenomena promoted by iTBS, were evaluate at baseline (t0), one week after (t1) and at 3-month follow-up.

hours [20,26]. During the 5 days of the treatment period, all patients received 1.5 h per day of training of the affected arm in the laboratory [27]. Training tasks were designed to force an intensive use of the paretic extremity, while requiring a progressive improvement of the quality of movement [28]. Participants were supervised by study staff who helped to direct them throughout the entire period the hand mitt was worn. Nine different shaping tasks were used during this 1.5-h period, which included buttoning a shirt, pouring water, and folding towel.

As part of experiment 2, patients were evaluated at baseline (t0), one week after (t1) and at 3-month follow-up. In these patients, we also explored motor cortex excitability of the AH and UH and the effects of the treatment (Bilateral tDCS + CIMT) on the propensity of the motor cortex of the AH to undergo LTP-like phenomena promoted by using iTBS [19].

Transcranial magnetic stimulation measurements of brain excitability (experiment 2)

Magnetic stimulation was performed with a high-power Magstim 200 (MagstimCo., Whitland, Dyfed). A figure-of-eight coil with external loop diameters of 9 cm was held over the motor cortex at the optimum scalp position to elicit MEPs in the contralateral first dorsal interosseous muscle (FDI). The induced current flowed in a postero-anterior direction.

We evaluated the threshold and amplitude of MEPs of AH and UH. The resting motor threshold (RMT) was defined as the minimum stimulus intensity that produced a liminal MEP (about 50 μV in 50% of 10 trials) at rest. The active motor threshold (AMT) was defined as the minimum stimulus intensity that produced a liminal MEP (about 200 μV in 50% of 10 trials) during isometric contraction of the tested muscle. The MEP amplitude was evaluated using a stimulus intensity of 120% RMT with the muscle at rest. Subjects were given audio-visual feedback of the electromyographic (EMG) signal at high gain to assist in maintaining complete relaxation; trials contaminated by EMG activity were discarded. Ten data sweeps were collected, and the peak-to-peak amplitude of the MEPs was calculated.

Intermittent theta burst stimulation

At t0 (baseline), t1 (1 week later) and at t2 (3-month follow-up), iTBS was delivered to the AH over the motor cortex "hot spot" for MEPs in the contralateral FDI muscle using a MagPro stimulator (Medtronic A/S Denmark) connected to a figure-of-eight coil (MCF B65). The magnetic stimulus had a biphasic waveform with a pulse width of about 280 μs and a maximum magnetic field strength of 1.5 T. The initial direction of the current induced in the brain was anterior to posterior. The exploited stimulation intensity was set to the 80% of AMT. We used the iTBS protocol in which 10 bursts of high frequency stimulation (3 pulses at 50 Hz) are applied at 5 Hz every 10 s, for a total of 600 pulses.

We evaluated the effects of iTBS, applied over M1 of AH, on MEPs amplitude elicited stimulating both hemispheres. MEP amplitude was evaluated before and after iTBS using the same stimulus intensity (120% RMT measured under baseline conditions).

Statistical analysis

Analyses were performed using SPSS 19 statistical software (IBM). The analysis of experiment 1 evaluated whether the clinical outcome at 1 week was influenced by neuromodulation alone (*Real_tDCS* vs *Sham_tDCS*).

Table 3
Clinical data for the four groups at multiple time points (mean and standard error of the mean – SER).

	Experiment 1								Experiment 2											
	tDCS_sham				*tDCS_real*				*tDCS real*						*tDCS sham*					
	t0		t1		t0		t1		t0		t1		t2		t0		t1		t2	
	Mean	SER	Mean	SER	Mean	SER	Mean	SER	Mean	SER	Mean	SER	Mean	SER	Mean	SER	Mean	SER	Mean	SER
NIHSS	7.00	1.35	3.71	0.97	7.29	2.01	4.00	0.85	5.80	0.94	2.40	0.31	1.33	0.44	5.90	0.66	3.80	0.65	1.89	0.54
mRS	3.57	0.43	3.00	0.49	3.57	0.20	3.00	0.38	3.30	0.26	2.40	0.37	0.44	0.29	3.80	0.20	3.10	0.46	1.11	0.35
ARAT	25.57	9.99	35.00	9.57	23.29	6.90	34.29	7.91	28.00	7.08	47.20	4.93	56.44	0.44	28.00	6.13	45.00	5.64	54.67	1.67
GRIP	0.19	0.12	0.34	0.12	0.26	0.11	0.37	0.13	0.17	0.10	0.38	0.09	0.67	0.12	0.22	0.09	0.32	0.10	0.67	0.13
MAL (AOU)	1.86	0.70	2.59	0.79	0.85	0.27	1.60	0.45	1.48	0.46	2.78	0.53	4.63	0.14	1.17	0.31	2.53	0.42	4.45	0.26
MAL (QOM)	2.00	0.74	2.54	0.78	0.89	0.27	1.56	0.43	1.63	0.48	2.85	0.53	4.53	0.16	1.39	0.39	2.58	0.40	4.37	0.23
NHPT	0.25	0.12	0.37	0.15	0.09	0.06	0.21	0.12	0.12	0.06	0.36	0.09	0.77	0.04	0.18	0.08	0.38	0.10	0.74	0.07

NIHSS = National Institutes of Health stroke scale, mRS = modified Rankin Scale, ARAT = Action Research Arm Test, GRIP = handgrip strength, MAL (AOU) = Motor Activity Log Rating Scale- Amount of Use, MAL (QOM) = Motor Activity Log Rating Scale – Quality of Movement, NHPT = 9 Hole Peg Test.

The analysis of experiment 2 addressed the effects of tDCS administration as add-on to CIMT at 1 week and up to three months on: i) clinical outcome; ii) brain excitability and inter-hemispheric balance; iii) iTBS-promoted cortical plasticity.

When only a value for each cell of the design was available, we used the analysis of variance or, if data did not fulfill the underlying assumptions, the Mann–Whitney test. By applying a General Estimating Equation (GEE) we have been able to exploit the several MEP values available for each patient in each cell of the design. Since MEPs were registered consecutively, we chose the autoregressive (lag = 1) working correlation within subjects [22].

In order to assess whether tDCS can modify the inter-hemispheric excitability asymmetry we decided to emphasize inter-hemispheric differences by computing the Laterality Index (LI), parameter that has been widely tested in stroke patients by fMRI investigations [29,30]. It corresponds to the following formula:

$$LI = \frac{(MEP_{UH} - MEP_{AH})}{(MEP_{UH} + MEP_{AH})}$$

LI ranges from −1 to +1 and the bigger the distance from 0, the higher is the inter-hemispheric imbalance. Positive values denote higher excitability of the UH.

In healthy subjects, LI tends to 0, with slightly difference due to the hemisphere dominance. In stroke patients it is considerably positive and, according to the inter-hemispheric competition model, tend to return to 0 in well recovered subjects [30]. We studied the LI differences between the two groups (*Real_tDCS* and *Sham_tDCS* – experiment 2). In particular, we computed LI differences normalized for the LI of second term of the difference (t1 – t0_norm, t2 – t1_norm and t2 – t0_norm). For instance, t1 – t0_norm corresponds to (LIt1 – LIt0)/LIt0.

Data distribution was studied by means of Kolmogorov–Smirnov test and proper transformations to better approximate Gaussianity and reduce outliers were applied when useful. The significance level was set to $P < 0.05$. The alpha-inflation due to multiple comparisons was faced according to Bonferroni-Sidak's procedure.

Results

Clinical outcome

Experiment 1 – For each clinical measure (NIHSS, mRS, ARAT, NHPT, NIHSS, MAL (AOU), MAL (QOM)) we computed a repeated measure ANOVA with *Time* as within subject factor (2 levels: baseline, 1 week) and *tDCS* (two levels: real and sham) as between subject factors. tDCS had no effect on clinical outcome (tDCS and the tDCS by Time interaction: $P > 0.200$ consistently). We confirmed the expected and well-known time-related clinical improvement (factor *Time* $P < 0.001$ for all the measures considered) (Table 3).

Experiment 2 – The repeated measure ANOVA with Time as within subject factor (3 levels: base, 1 week, 3 months) and tDCS (2 levels: real and sham) as between subject factor showed the absence of tDCS effect on clinical outcome (factor tDCS and tDCS by Time interaction: $P > 0.050$ consistently).

Cortical excitability – experiment 2

The aim of the analysis was to check whether there was a change of brain excitability across *hemispheres*, *time* and *groups*.

The two groups (*real tDCS* vs. *sham tDCS*) had no different excitability at baseline (t0), as demonstrated by the lack of tDCS factor effect (Wald chi-square = 0.352, df = 1, $P = 0.553$) and of Hemisphere by tDCS interaction (Wald chi-square = 066, df = 1, $P = 0.797$). The inter-hemispheric excitability imbalance, well-known in acute stroke, was confirmed in both groups by the presence of a main effect of Hemisphere (Wald chi-square = 15.334, df = 1, $P < 0.001$).

The further step was to compare the excitability of the two groups (real tDCS and Sham tDCS) along the time-course of the investigation computing a GEE model with Patient as "Subject" (or cluster) variable, MEP values as dependent variable and Hemisphere (AH and UH), Time (t0, t1, t2) and tDCS (Real and Sham) as predictors. The presence of a significant triple interaction Time by Hemisphere by tDCS (Wald chi-square = 31.652, df = 7, $P < 0.001$) showed that the inter-hemispheric balance of excitability is differently modulated along time accordingly with tDCS exposure (*real tDCS* and *Sham tDCS*) (Fig. 2A).

In the context of the effect of tDCS on the inter-hemispheric balance, the *Real_tDCS* group, compared to the *Sham_tDCS* group, showed a reduction of LI when considering both t2 – t1_norm ($P = 0.007$) and t2 – t0_norm ($P = 0.015$). No differences between groups were found considering t1 – t0_norm (Fig. 2B).

In summary, the main effect of tDCS was the reduction of inter-hemispheric imbalance at 3 months of follow-up (Table 4).

LTP/LTD-like changes promoted by iTBS – experiment 2

We wanted to evaluate whether the addition of tDCS to a CIMT protocol can change the amount of LTP of the AH and of LTD of UH promoted by iTBS applied over the AH. We computed a GEE model with Patient as "Subject" (or cluster) variable, MEP values as

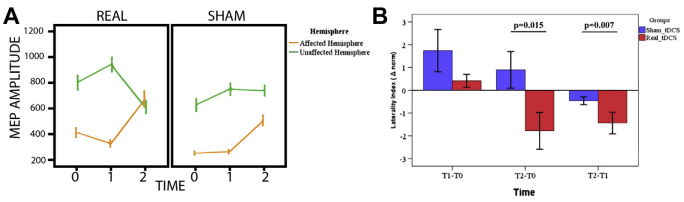

Figure 2. Effect of tDCS on cortical excitability and inter-hemispheric balance. A: Cortical excitability (MEP amplitude mean ± SEM) by Group (*left panel*: Real_tDCS vs *right panel*: Sham tDCS) and Hemispheres (orange: Affected hemisphere vs green: Unaffected hemispheres). B: Normalized difference of Laterality Index (LI) (mean ± SER) across multiple time points (t1 − t0 = LI at 1 week − LI baseline; t2 − t0 = LI at 3 months − LI baseline; t2 − t1 = LI at 3 months − LI at 1 week) for the two groups (Sham_tDCS − blue and Real_tDCS − red). In detail, LI differences are normalized for the LI of second term of the difference (t1 − t0_norm, t2 − t1_norm and t2 − t0_norm): for instance, t1 − t0_norm corresponds to (LIt1 − LIt0)/LIt0. All MEP amplitudes are measured pre-iTBS.

dependent variable and Hemisphere (AH and UH), tDCS (Real and Sham), Time (t0, t1, t2) and TBS (Pre-TBS and Post-TBS) as predictors.

The presence of a significant Hemisphere by Time by TBS by tDCS interaction (Wald chi-square = 41.472, df = 14, $P < 0.001$) suggests that the effect of TBS on each hemisphere differently changes along time in the two groups.

Once the GEE model with Hemisphere (AH and UH), Time (t0, t1, t2) and TBS (Pre-TBS and Post-TBS) as predictors was applied separately for each group, we found a significant Hemisphere by Time by TBS interaction only for the *Real_tDCS* group (*Real_tDCS*: Wald chi-square = 28.870, df = 7, $P < 0.000$; *Sham_tDCS*: Wald chi-square = 6.350, df = 7, $P = 0.499$). Therefore, in other words, the "Real tDCS" supports a different modulation of TBS effects across Hemispheres and Time. The visual inspection of the curves (Fig. 3) suggests that at t1 iTBS produced both greater increase of AH excitability and decrease of UH excitability in patients exposed to *Real_tDCS*.

Finally, the post-iTBS study of LI suggests that real tDCS decreases inter-hemispheric imbalances compared to Sham_tDCS (t2 − t1_norm: Mann–Whitney test, $P = 0.042$, not corrected for multiple comparisons) (Fig. 4). In other words, tDCS improves baseline inter-hemispheric balance and also TBS effects on inter-hemispheric balancing.

Discussion

Our first important conclusion, from the assessment of all clinical instruments in both experiments, is that, in acute stroke, our strategy of tDCS does not result in significant clinical improvements, at 1 week and/or at three months, due to five-daily bilateral tDCS sessions, either administered alone or in association with CIMT, beyond the ones achieved by the physical therapy alone or through the spontaneous recovery.

However, it might be the case that the clinical instruments, or the limited follow-up, are not sensitive enough to index potential clinical changes. The idea for this study was based on two hypotheses: 1) tDCS, as a plasticity-enhancing intervention, would facilitate motor-relearning in acute stroke patients enhancing motor recovery; 2) accordingly to the inter-hemispheric competition model, bilateral stimulation with anodal tDCS over the AH and cathodal tDCS over the UH could promote the balance of excitability between the two hemispheres, with positive effects on functional recovery [8].

In order to investigate deeper these hypotheses, in experiment 2, we added to the evaluation of the clinical outcome the assessment of the asymmetry of excitability and of the level of plasticity before and after iTBS, carried on at different time points after stroke. The results showed that tDCS has an effect on cortical excitability balancing: the *Real_tDCS* group undergoes a clear modulation of AH–UH balance of excitability, to such an extent that, at three months, no differences between the UH and AH excitability (Fig. 2A) were found. This aspect is further confirmed by the study of the LI changes along time: considering as reference point both t0 (baseline) and t1 (1 week), at t2 (3 months) the *Real_tDCS* group shows a reduction of AH-UH imbalance significantly different from the *Sham_tDCS group* (Fig. 3).

As regard the interaction of *Real_tDCS* with the TBS-promoted plasticity, we report an enhancement of the AH LTP-like and UH LTD-like activity and, considering the LI post-iTBS, a significant reduction of the imbalance (Table 4).

The decrease of the inter-hemispheric imbalance due to the combination of CIMT with real tDCS was more evident at 1 week as indexed by the propensity to undergo LTP-like plasticity and at the 3-month for the changes in excitability. Remarkably, this difference in the time-course of the effects is compatible with the earlier induction of a tDCS-dependent transient modulation of plasticity that need months to produce consolidated changes and thus to become evident on a more direct measurable functional index of the motor corticospinal activity (MEP amplitude).

In summary, although we did not find additional clinical improvement associated with tDCS, we did find consistent changes

Table 4
Mean, median and SER of Laterality Index for Real_tDCS and Sham_tDCS groups at multiple time points and normalized differences between time points.

Time	Real_tDCS						Sham_tDCS					
	PRE_iTBS			*POST_iTBS*			*PRE_iTBS*			*POST_iTBS*		
	Mean	Median	SEM	Mean	Median	SEM	Mean	Median	SEM	Mean	Median	SEM
t0	0.287	0.146	0.122	0.086	0.126	0.042	0.251	0.105	0.126	0.077	0.092	0.030
t1	0.278	0.137	0.122	0.169	0.092	0.110	0.279	0.126	0.123	0.085	0.077	0.039
t2	−0.001	0.006	0.0254	−0.001	0.004	0.018	0.041	0.045	0.017	0.038	0.039	0.017
t1 − t0_norm	0.415	0.143	0.285	−0.180	−0.168	0.352	1.740	0.646	0.930	−0.691	−0.072	0.858
t2 − t0_norm	−1.781	−0.992	0.806	−1.052	−0.960	0.318	0.895	−0.270	0.806	1.182	−0.556	2.211
t2 − t1_norm	−1.440	−0.992	0.476	−1.052	−1.000	0.249	−0.461	−0.497	0.173	2.606	−0.474	3.241

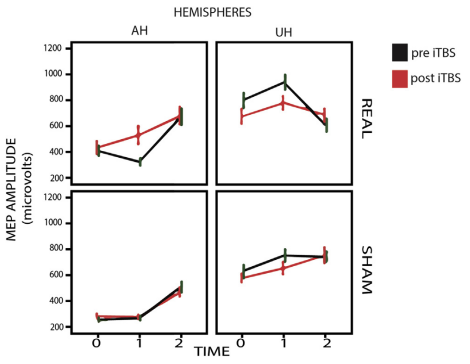

Figure 3. Effect of tDCS on LTP-LTD like phenomena iTBS-dependent. A 2×2 matrix describing the triple interaction on tDCS condition × Hemisphere × Time on cortical excitability (MEP amplitude (microvolts) – mean ± SEM). Rows: Groups (panels in the upper row: Real_tDCS vs panels in the lower row: Sham tDCS), Columns: Hemispheres (first column: Affected hemisphere vs second column: Unaffected hemispheres). Color code differentiates values relative to Pre-iTBS (black) vs Post-iTBS (red) at each time point (t0 = baseline, t1 = 1 week, t2 = 3 months).

in neurophysiological parameters; thus, it is worth assessing which factors may be responsible for the dissociation between the positive neurophysiological results and clinical improvement.

In chronic stroke patients, several studies reported that five to ten days of bihemispheric tDCS in combination with motor rehabilitation (i.e. CIMT) are able to achieve significant clinical gain [20,31] and in parallel, as in the present study, a reduction of the inter-hemispheric inhibition from the UH to the AH [20]. Moreover, in the same paradigm, Lefebvre et al., found in the real tDCS group not only better learning performance, but also higher retention abilities including transferring learned skill into new, previously untrained, tasks [32]. Conversely, and in line with the present study, Rossi and co-workers, that enrolled acute stroke patients, showed that five-daily sessions of anodal tDCS applied to the motor cortex of the AH appear to be safe, but do not improve clinical outcomes [33]. It looks like that something differentiates acute and chronic stroke regarding the ability to respond with improved clinical outcome to tDCS. It could be argued that, in acute stroke, the level of plasticity is significantly dysfunctional or it already reaches a ceiling effect that does not allow five days of tDCS to produce additional evident clinical benefits, thus longer treatments may be needed. Accordingly, our data showed a congruent positive trend, although not-significant, for all the clinical scores at 3 month follow-up and Reis et al., showed, in healthy, that the only advantage in motor learning given by tDCS added to motor training was detectable not before than 3-month after the intervention [34].

Moreover, the visual inspection of the pattern of changes of excitability from 1 week to three months (Fig. 2A) reveals that the real tDCS contribution to re-establishing the balance is mostly due to an inhibition of UH. It can be speculated that, in the acute phase of stroke, the AH may be not able to respond properly to neuromodulation, either because of excessive damage or alternatively because of saturation of plasticity induced by the damage itself and the spontaneous recovery. Along this line, in chronic stroke patients, given that the recovery of paretic hand motor function is mostly associated with plasticity of the AH; this may account for the lack of clinical improvements in this study. Indeed, when cathodal tDCS of UH, together with motor training, was associated not only with an inhibition of the UH, but also with disinhibition of the AH; an improvement in the ability to retain new motor skills was observed. In addition, this clinical improvement was correlated with changes of AH [35].

The rationale employed for tDCS setting (cathode over AH and anode over UH) is based on the inter-hemispheric competition model [36], however, it is possible that in the acute phase, the enhanced excitability of UH can be alternatively considered as compensatory (*vicariation model* [29]) and thus its inhibition may be detrimental [37]. Although this model is based on TMS-indexed excitability changes, the correlation between excitability changes and behavior may not be true and in fact may change according to the phase of stroke. Thus results should be interpreted carefully given this possibility.

A further factor that may be associated with the absence of clinical improvements is the correct selection of the tDCS parameters of stimulation. Indeed, tDCS is commonly considered excitatory or inhibitory depending only on the polarity of the electrode placed over the target area. However, for instance, the duration of stimulation, and the interval between following sessions, have been showed to be key factors in determining the direction [38] and the magnitude of the after-effect [39,40].

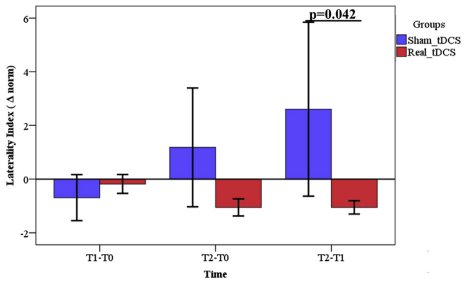

Figure 4. Laterality Index post-iTBS. Normalized difference of Laterality Index (LI) (mean ± SER) across multiple time points (t1 – t0 = LI at 1 week – LI baseline; t2 – t0 = LI at 3 months – LI baseline; t2 – t1 = LI at 3 months – LI at 1 week) for the two groups (Sham_tDCS – blue and Real_tDCS – red). In detail, LI differences are normalized for the LI of second term of the difference (t1 – t0_norm, t2 – t1_norm and t2 – t0_norm): for instance, t1 – t0_norm corresponds to (LIt1 – LIt0)/LIt0. All MEP amplitudes are measured post-iTBS.

Conclusions

This study shows two important novel findings: (i) the addition of bilateral tDCS to the CIMT significantly reduces inter-hemispheric imbalance between AH and UH as indexed by TMS induced neurophysiologic outcomes and this effect is maximal at three-month follow-up; (ii) tDCS does not lead to additional clinical benefits in acute stroke despite the positive neurophysiologic findings. However, inter-hemispheric imbalance is widely considered as an important predictor of poor rehabilitation outcomes at the chronic phases of stroke. Then, it may be argued that the neurophysiologic findings of this study would have a higher impact on the clinical outcome evaluated in the chronic, rather than the acute, phases of stroke. Future studies aiming to respond to questions not answered by the present study are needed, in order to determine, for instance, the relative contribution of GABA and glutamate in the described changes of excitability.

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Paper 4

Neuroscience Letters 553 (2013) 148–152

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Inhibitory theta burst stimulation of affected hemisphere in chronic stroke: A proof of principle, sham-controlled study

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HIGHLIGHTS

- We evaluated if inhibitory modulation of stroke hemisphere can enhance recovery.
- We found no deleterious effects of inhibitory stimulation on recovery.
- All patients showed some improvement from a retraining protocol for the upper limb.
- Inhibition of stroke hemisphere is safe and has the potential to enhance recovery.

ARTICLE INFO

Article history:
Received 19 April 2013
Received in revised form 22 June 2013
Accepted 8 August 2013

Keywords:
Transcranial magnetic stimulation
Stroke
LTP
LTD
Rehabilitation

ABSTRACT

Non-invasive brain stimulation is presently being tested as a potential therapeutic intervention for stroke rehabilitation. Following a model of competitive interactions between the hemispheres, these interventions aim to increase the plasticity of stroke hemisphere by applying either excitatory protocols to the damaged hemisphere or inhibitory protocols to the non-stroke hemisphere. Here we test the safety and feasibility of using an inhibitory protocol on the stroke hemisphere to improve the response to conventional therapy via a homeostatic increase in learning capacity. Twelve chronic stroke patients received TBS to stroke hemisphere (6 patients inhibitory TBS and 6 sham TBS) followed by physical therapy daily for 10 working days. Patients and therapists were blinded to the type of TBS. Action Research Arm Test (ARAT), Nine-Hole Pegboard Test (NHPT) and Jebsen–Taylor Test (JTT) were the primary outcome measures, grip and pinch-grip dynamometry were the secondary outcome measures. All patients improved ARAT and JTT scores for up to 3 months post-treatment. ARAT scores improved significantly in both real and sham groups, but only patients receiving real TBS significantly improved on the JTT: 3 months post-treatment mean execution time was reduced compared to baseline by 141 s for real group and by 65 s for the sham group. This small exploratory study suggests that ipsilesional inhibitory TBS is safe and that it has the potential to be used in a larger trial to enhance the gain from a late rehabilitation program in chronic stroke patients.

1. Introduction

Non-invasive human brain stimulation in the form of repetitive transcranial magnetic stimulation (rTMS) or transcranial direct current stimulation (tDCS) can induce long-lasting changes in the excitability of central motor circuits via long-term potentiation/depression (LTP/LTD)-like phenomena that share major properties of LTP/LTD described at cellular level [18]. Several recent studies tested whether induction of LTP-like effects in the stroke hemisphere can enhance the effects of motor rehabilitation after stroke [10,16]. The hypothesis is that stimulation facilitates the stroke hemisphere and initiates changes in synaptic plasticity that improve therapy by enhancing learning-related changes in synaptic connections that are required for reacquisition of skills [17]. Conversely, inhibitory stimulation of the non-stroke hemisphere might reduce its excitability and reduce transcallosal inhibition of stroke hemisphere, with the same consequences for learning. Several clinical studies reported some positive effects from repeated sessions of brain stimulation [1], however, the effects were limited and variable.

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http://dx.doi.org/10.1016/j.neulet.2013.08.013

Recent work has, however, suggested that rTMS could improve learning via a different mechanism that involves the phenomenon of "homeostatic" plasticity. This postulates that the ease of producing synaptic LTP/LTD depends on the prior history of neural activity. The greater the activity the more difficult it is to induce LTP; whereas LTD is more difficult to induce with a history of low activity. Homeostatic-like interactions have been reported in the human brain using a variety of brain stimulation protocols [12]. For example, a protocol capable of inducing LTD-like effects strongly facilitates motor learning while protocols inducing LTP-like effects have a less pronounced and short-lived facilitatory effect on learning [12]. In the context of stroke this would predict that, contrary to present practice which uses excitatory protocols, an inhibitory rTMS protocol that induces LTD-like effects on the stroke hemisphere would lead to better relearning in stroke patients through mechanisms of homeostatic metaplasticity [12].

We designed a proof-of-principle double blinded semi-randomised sham-controlled trial to assess the safety and potential efficacy of this approach by measuring whether clinically important long-lasting differences can be achieved by adding continuous theta burst stimulation (cTBS) of the lesioned hemisphere to a standardized physiotherapy protocol for the upper limb in chronic stroke. CTBS is a robust form of inhibitory rTMS; its after-effects, thought to be due to LTD-like changes [8], can last up to 1 h, an excellent time window for a therapy session. We hypothesized that immediate and long-term outcomes of the active treatment would be significantly better than sham treatment.

2. Subjects and methods

2.1. Subjects

12 chronic stroke patients gave their written informed consent for the study which was performed according to the Declaration of Helsinki and approved by the local ethics committee. Inclusion criteria were: (a) first-ever ischemic stroke at least 1 year earlier; (b) moderate residual hand function, defined as grasp strength $\geq$1% of the unaffected hand, preserved extension at the wrist ($\geq$20°), and baseline score in Nine Hole Pegboard Test (NHPT) $\leq$70% of the unaffected hand; (c) ability to give informed consent and comprehend instructions. Exclusion criteria were: (a) significant spasticity (Ashworth score >2); (b) patients not able to perform dynamometry; (c) concomitant neurological conditions, including any history of epilepsy and significant comorbidities; (d) cognitive impairment or any substantial decrease in alertness, language reception, or attention that might interfere with understanding instructions for motor testing; (e) apraxia; (f) excessive pain in any joint of the paretic extremity; (g) contraindications to TMS such as metal head implants; (h) advanced liver, kidney, cardiac or pulmonary disease; (i) history of significant alcohol or drug abuse; (l) depression or use of neuropsychotropic drugs such as antidepressants or benzodiazepines. The National Institute of Health Stroke Scale (NIHSS) and the Barthel Index (BI) were used to evaluate neurological impairment and disability at the enrolment.

2.2. Primary outcome measures

Since this was an exploratory trial in which we aimed to evaluate changes in global hand function, we chose 3 primary outcome measures that evaluate different aspects of that. These were Action Research Arm Test (ARAT; score 0–57), Jebsen-Taylor Test (JTT) and Nine Hole Pegboard Test (NHPT).

(1) ARAT is a broad measure of upper extremity function in patients with focal disability [7].

(2) Jebsen–Taylor Test (JTT) has been shown to be valid and reliable in the normal population [11] and in chronic stroke patients [5,9]. The modified version used here has 6 subsets. Items were tested 5 times at each assessment. The time in seconds to complete each subset was recorded: the maximal amount of time allotted for each item was 120 s so that 120 s were assigned to the tasks that could not be concluded [4]. The hands were tested alternately. Since performance stabilizes after 2–3 trials, only the last two trials were averaged and used for analysis. However, to better characterize performance in patients who were not able to perform any of the JTT tasks at baseline, we used the method of calculation performed in one of our previous studies about hand function in chronic stroke patients [14]. Thus scores were normalized to the performance of unaffected hand and computed as follows: cannot do or <0.05 = 1, 0.05–0.09 = 2 0.1–0.14 = 3, and so on; thus, the range was 1–20, each point reflecting an improvement of 5% of the maximum score that is the score of the unaffected hand. The items were then summed to produce a JTT total score (range 6–120, 11.4 points reflecting 10% improvement) [14].

(3) NHPT is a test sensitive to changes in finger dexterity [6]. Each hand was tested alternately for 3 times, starting from the paretic one. Sixty seconds were allowed for each single attempt: if not completed, the number of pegs placed in 60 s was recorded. Final scores were computed as the ratio pegs/s placed by the paretic hand, averaged over 3 trials and normalized to the average score of the unaffected hand (range 0–1; 0, cannot do).

2.3. Secondary outcome measures

Grasp and pinch grip dynamometry were performed using a digital dynamometer (Biometrics Ltd, Newport, UK). Each patient was instructed to perform 3 attempts at grip and pinch, alternating the hands. Maximal grip strength, when normalized to the unaffected hand, is highly reproducible in chronic stroke patients [2].

2.4. Motor cortex excitability

We evaluated changes in motor cortex excitability in a subgroup of patients [4 in the real group (mean age: 59.5 ± 11.7 (SD) years) and 4 in the sham group (age: 56.7 ± 16.1; $p = 0.5$)] of both affected (AH) and unaffected (UH) hemisphere at baseline, T1 and T2.

AMT was evaluated for all the patients of the real group at each time point to set the intensity for cTBS.

Magnetic stimulation was performed with a high-power Magstim 200 (MagstimCo., Whitland, Dyfed). A figure-of-eight coil with external loop diameters of 9 cm was held over the motor cortex at the optimum scalp position to elicit MEPs in the contralateral first dorsal interosseous muscle (FDI). The induced current flowed in a postero-anterior direction.

For both AH and UH, we evaluated active (AMT) and resting (RMT) motor threshold and amplitude of motor evoked potentials (MEPs). MEPs were band pass filtered (bandwidth 3 Hz–3 kHz) (Digitimer D360 amplifiers) and each single trial was recorded on computer for later analysis using a CED 1401 A/D converter (Cambridge Electronic Design, Cambridge, UK) and associated software. The responses to 20 stimuli obtained at rest at an intensity of 120% RMT were averaged.

2.5. Interventions

Real or sham brain stimulation, followed by physical therapy targeting the arm, was delivered daily for 10 consecutive working days.

2.5.1. Transcranial brain stimulation

rTMS was applied over the hand motor area of the AH using a MagPro stimulator (Medtronic A/S, Denmark) and a figure-of-eight shaped coil (MCF-B65) with the handle pointed posteriorly and approximately perpendicular to the central sulcus.

Active rTMS used continuous Theta Burst Stimulation (cTBS) in which 3 pulses are given at 50 Hz, repeated every 200 ms for a total of 600 pulses. Stimulation intensity was 80% active motor threshold (AMT), defined as the minimum single pulse intensity required to produce a motor evoked potential greater than 200 μV on more than five out of ten trials from the contracted contralateral first dorsal interosseous muscle. CTBS at this intensity suppresses cortical excitability reaching a maximum effect at about 5–10 min after the end of the stimulation [9]. Affected hemisphere AMT was determined at each session to adjust cTBS intensity.

Sham rTMS was performed using the same stimulator connected to the placebo coil (MCF-P-B65) which has no stimulating effect but produces similar auditory and tactile sensations as the active coil. The site of stimulation and the number of stimuli were identical to those used for the active rTMS protocol. Sham TBS intensity was arbitrarily set at 50% of MSO.

2.5.2. Physical therapy

Physical therapy included strength training for the wrist, fingers and thumb and grasp and repetitive task practice; the latter aimed mainly at hand function, including, however, proximal elements through functional reach to different areas within the work space. It was designed to ensure the same intensity of intervention independent of baseline functional ability, as described in detail in an earlier publication [15]. Each session lasted approximately 1 h. Therapy was given by a single certified physiotherapist.

3. Experimental design

All the patients received physical therapy. They were randomized to active ($n = 6$, mean age: 59.5; SD: 12.4; range: 46–75) or sham cTBS ($n = 6$; mean age: 57.5; SD: 12.6; range: 39–76) through a randomization stratification approach. We stratified the groups by using NIHSS, BI and ARAT at baseline to ensure that both groups had a similar distribution regarding degree of impairment.

Researcher randomizing patients and researchers delivering cTBS were not involved in outcome assessments and data analysis; the physiotherapist, patients and researchers involved in data analysis were blind to the type of cTBS delivered (i.e. sham or real), in order to obtain a double-blinded sham-controlled study design.

ARAT, NHPT and JTT were evaluated at baseline (T0) and 4 days (T1), 1 month (T2) and 3 months (T3) after the end of the treatment; cortical excitability, grasp and pinch strength were evaluated at T0, T1 and T2 (Fig. 1).

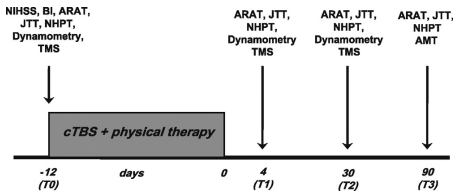

Fig. 1. Study design. Timing of clinical evaluations with respect to the end of treatment. Treatment (real/sham cTBS + physical therapy) was delivered for 10 consecutive working days. Baseline evaluation was performed in the first day of treatment ARAT: Action Research Arm Test; JTT: Jebsen–Taylor Test; NHPT: Nine Hole Pegboard Test; TMS: transcranial magnetic stimulation (motor cortex excitability study); cTBS: continuous Theta Burst Stimulation.

Table 1
Demographic and clinical characteristics of the patients at baseline.

	cTBS ($N = 6$)	Sham ($N = 6$)	p
Age (years)[a]	59.5 ± 12.4	57.5 ± 12.3	0.78
Sex (M/F)	3/3	4/2	0.77[c]
MSS (months)[a]	34.8 ± 17.5	30 ± 27.6	0.7[d]
Lesion location			
Subcortical	0/6	1/6	
Cortical involvement[e]	6/6	5/6	
Lesion side (% dominant)	1/6	2/6	0.85[c]
Barthel index[a]	16.5 ± 3.27	18.3 ± 1.3	0.24[d]
NIHSS[a]	3.1 ± 1.5	4.0 ± 1.4	0.34[b]
ARAT[a]	21.8 ± 8.6	23.6 ± 10.2	0.74[b]
NHPT (ah/hh ratio)[a]	0.03 ± 0.03	0.02 ± 0.02	0.33[b]
JTT (normalized scores; ah)[a]	17.5 ± 8.9	19.0 ± 14.9	0.83[b]
JTT (s; ah)[a]	415 ± 184	416 ± 247	0.99[b]
Grasp (ah/hh ratio)[a]	0.5 ± 0.1	0.33 ± 0.16	0.06[b]
Pinch grip (ah/hh ratio)[a]	0.3 ± 0.15	0.45 ± 0.31	0.32[b]

[a] Mean ± standard deviation.
[b] t-Test.
[c] χ^2-Test.
[d] Mann–Whitney.
[e] Sparing the primary motor cortex.
MSS: months since stroke; NIHSS: National Institute of Health Stroke Scale; ARAT: Action Research Arm Test; NHPT: Nine-Hole Pegboard Test; JTT: Jebsen–Taylor Test; ah: affected hand; hh: healthy hand.

4. Statistics

We analyzed ARAT, NPHT, JTT time and JTT normalized scores using separate repeated measures ANOVA with TIME (T0, T1, T2 and T3) as within-subject factor and GROUP (real vs sham cTBS) as between-subject factor; grasp and pinch strength were analyzed in the same way but with only 3 time points (T0, T1 and T2). When significant main effects or interactions were found, post hoc paired two tailed t-tests were performed. The level of significance was set at $P < 0.05$: both for primary and secondary outcome measures we performed correction for multiple comparisons by Bonferroni method, so that p value was set at 0.017.

Before entering the data in ANOVA, we performed Kolmogorov–Smirnov (KS) test to check for normal distribution.

5. Results

There were no adverse events. Groups were balanced at baseline for age, time from the acute event, NIHSS, BI, lesion side, M/F ratio and residual hand function as evaluated by ARAT and JTT baseline scores (Table 1).

KS test revealed that all the data were normally distributed.

5.1. Primary outcome measures

5.1.1. ARAT

Repeated measures ANOVA showed a significant effect of TIME ($F_{3,10} = 29.4$; $p < 0.001$), but no significant TIME × GROUP interaction ($F_{3,30} = 0.16$; $p = 0.9$). Thus, physical therapy was effective regardless the rTMS intervention. Nevertheless, we explored the effect of TIME in more detail by a within-group comparison examining changes from baseline in each group (real- and sham-cTBS) with paired t-tests. Both real- and sham-cTBS groups improved significantly at T1, T2 and T3 (Fig. 2). Patients undergoing real cTBS increased mean ARAT score above baseline by about 30% at T1 and by about 36% at T2, T3. The sham group increased about 27% at T1, about 30% at T2 and about 34% at T3.

5.1.2. JTT

Repeated measures ANOVA for JTT execution time showed a significant effect of TIME ($F_{3,10} = 10.5$; $p < 0.0001$) and a significant

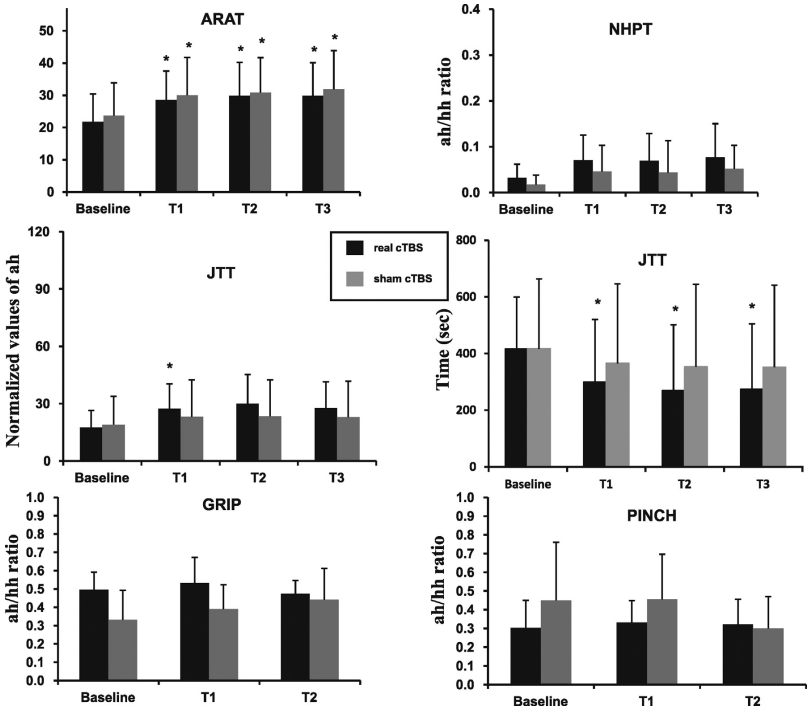

Fig. 2. Mean scores in upper limb function tests before and after intervention. Error bars represent standard deviations; *$p < 0.017$, comparisons were done always toward baseline in the same group (i.e. T1 vs baseline, T2 vs baseline, T3 vs baseline). ah: affected hand; hh: healthy hand.

TIME × GROUP interaction ($F_{3,30} = 3.173$; $p = 0.03$). This simple measure of the time taken to perform the JTT might not provide a good description of the data if patients cannot perform any of the tasks at baseline, but then succeed after treatment. To account for this, we also used a normalized score in which we express the performance of the affected side as a percentile of the performance of the unaffected side (see Ref. [16] for further details). A repeated measures ANOVA on these normalized scores showed a significant effect of TIME ($F_{3,10} = 8.84$; $p = 0.0002$) but no significant TIME × GROUP interaction ($F_{3,30} = 1.9$; $p = 0.1$). However, a within-group comparison (both for performance time and normalized scores) revealed that only the patients who underwent real cTBS improved at T1, T2 and T3 ($p = 0.01$, $p = 0.01$ and $p = 0.015$ respectively for performance time; $p = 0.01$, $p = 0.02$ and $p = 0.03$ respectively for normalized scores), whereas no significant effect of TIME was found in the sham group (Fig. 2). In patients undergoing real cTBS, mean JTT execution time was reduced compared to baseline by 116 s at T1, 147 s at T2 and 141 s at T3 whereas for the sham group the mean reduction in performance time was 51, 63 and 65 s at the same time points; furthermore normalized scores increased compared to baseline by about 55% at T1, 70% at T2 and 60% at T3, whereas for the sham group the mean increase was between 15% and 22% at the same time points. Between groups post hoc analysis revealed no significant differences at each time point for both measuring methods.

5.1.3. NHPT

There was a significant effect of TIME ($F_{3,10} = 5.42$; $p = 0.004$) but no significant TIME × GROUP interaction ($F_{3,30} = 0.108$; $p = 0.9$). A within-group comparison revealed that both the real- and the sham-cTBS groups had a similar but non-significant trend toward improvement at the three time points post-intervention (T1: real $p = 0.08$, sham $p = 0.16$; T2: real $p = 0.08$, sham $p = 0.27$; T3 real $p = 0.10$, sham $p = 0.10$) (Fig. 2).

5.2. Secondary outcome measures

5.2.1. Grasp and pinch grip dynamometry

ANOVA revealed no significant effect of TIME (grasp $F_{2,10} = 1.3$ $p = 0.29$; pinch $F_{2,10} = 1.03$, $p = 0.37$) nor significant TIME × GROUP interaction (grasp $F_{2,20} = 2.3$, $p = 0.12$; pinch $F_{2,20} = 1.1$, $p = 0.34$) (Fig. 2).

5.2.2. Motor cortex excitability

At baseline paired *t*-test revealed a significant difference in MEP amplitude between UH and AH in both subgroups [(UH vs AH): real group: 606 ± 240 μV vs 160 ± 177 μV ($p = 0.01$): sham group: 421 ± 205 μV vs 90 ± 75 μV ($p = 0.01$)]. No significant difference at baseline was disclosed in motor thresholds and MEP amplitude for both hemispheres between the two studied groups.

ANOVA revealed no significant effect of TIME nor significant TIME × GROUP interaction for both motor threshold and for MEP amplitude. No significant change in AMT was seen at different time points for both studied groups.

6. Discussion

This exploratory trial tested the idea that application of an inhibitory form of rTMS to the stroke hemisphere can improve the response to physical therapy via a homeostatic action on synaptic plasticity and hence on learning. Importantly, we found no deleterious effects of cTBS on recovery, as might have been expected if it had had the opposite effect to the presently used non-homeostatic protocols, since all patients showed some improvement in function. Patients in both real and sham groups achieved sustainable improvements in the ARAT, NHPT and JTT, illustrating the success of the therapy. However, only patients who underwent real cTBS had a significant improvement in JTT while both the real- and the sham-cTBS groups improved in the ARAT and NHPT. We observed a mean reduction in JTT performance time, when compared to baseline values, by 116 s at T1, 147 s at T2 and 141 s at T3 for the real group whereas for the sham group the mean reduction in performance time was 51, 63 and 65 s at the same time points. Furthermore, this difference between real and sham group was confirmed by a within-group comparison examining changes of JTT normalized scores from baseline in each group (real- and sham-cTBS). Although all patients improved on functional measures of motor ability, there was no change in handgrip strength. Nevertheless, since the data were similar in real and sham groups, it cannot be viewed as an adverse effect from pre-treatment with real-cTBS.

We hypothesize that cTBS, by reducing excitability and inducing LTD-like changes, might enhance and/or accelerate the training process and the effects of rehabilitation [13]. However, there is another possible explanation for the results. Bradnam et al. [3] suggested that in some patients, recovery of function is mediated by increased ipsilateral control from the non-stroke hemisphere. If this were the case, then inhibitory cTBS to the stroke hemisphere may remove interfering activity and allow the non-stroke hemisphere to improve its response to rehabilitation. Although possible, we think this unlikely since inhibition from the stroke hemisphere onto the non-stroke hemisphere should be much reduced, such that removal would have little effect on function. This point can be investigated by evaluating the excitability of the healthy and stroke hemisphere before and after treatment. In present study we showed no significant change in cortical excitability after the treatment both for the AH and UH. However, further studies evaluating also the changes in ipsilateral MEPs after stimulation of the healthy hemisphere are needed to better understand the contribution of ipsilateral pathways in the recovery.

There were no between-group differences in outcome measures other than JTT, perhaps because of the relatively small number of patients. Nevertheless it could be also associated with the specificity and sensitivity of the individual measures. For example the JTT, unlike other measures, improves with practice particularly in naive individuals [5,9]. This is why 5 trials were allowed at each assessment and only the last two ones were used for analysis. In our previous study, a clear learning curve was seen in the pre-treatment assessments but not in the post-treatment ones; in other words there was no further learning with practice after the treatment period (Talelli, unpublished data). If the intervention in this study creates an environment that facilitates practice-induced learning it is possible that patients continued improving in the JTT tasks during the 5 trials in the post-treatment assessments and thus achieved a higher plateau. The lack of significant variations in the other clinical scales could be due to an occlusion effect between TMS and rehabilitation for the functions explored by these scales, that is, the improvement produced by rehabilitation cannot be further enhanced by adding cTBS.

Previous studies combining standard rehabilitation with rTMS protocols that enhance ipsilesional excitability or suppress contralesional excitability were based on a non-homeostatic interaction of brain stimulation and motor training and their results have been conflicting and variable. Even though our results should be considered with caution, due to the limited number of patients included, cTBS used in a homeostatic protocol may prove to be another potential therapeutic intervention after stroke. Future studies could define the optimal number of cTBS sessions and the most appropriate time of intervention.

Acknowledgements

Contributing therapists: Cecilia Gillini.

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Results

Paper 1: Differential Effects of HRAS Mutation on LTP-Like Activity Induced by Different Protocols of Repetitive Transcranial Magnetic Stimulation

In Costello Syndrome (CS) patients mean baseline-normalized MEP am- plitude was increased by about 250% immediately after PAS and by approximately 320% when evaluated at 30 minutes after the end of PAS, whereas after iTBS we observed no significant change of MEP amplitude at the two time-points. In healthy subjects we observed an increase in baseline-normalized MEP amplitude by about 73% at T1 and by about 39% at T2 after PAS and an increase by about 51% at T1 and by about 40% at T2 after iTBS: post hoc unpaired t-test for comparison of the effects of the two stimulation protocols in healthy group did not reveal any significance, that means that iTBS and PAS produce the same facilitatory effect in baseline-normalized MEP amplitude of controls.

When CS were compared to controls, iTBS induced a significant increase in baseline-normalized MEP amplitude in controls, but not in CS patients and conversely we confirmed that PAS induced a significantly more pronounced increase of MEP amplitude in CS when compared to control group.

Paper 2: The Level of Cortical Afferent Inhibition in Acute Stroke Correlates With Long-Term Functional Recovery in Humans

AH-SAI and AH-MEP amplitude were lower than corresponding UH and control values. No evidence of association between electrophysiological parameters and stroke severity in the acute phase was found. Looking at correlations with clinical status at 6 months, the only significant associations were found with AH-SAI.

When the effect of AH-SAI on mRS was adjusted for the confounding effect of baseline clinical status (National Institutes of Health Stroke Scale at T0), the nonparametic partial correlation remained significant (rho 0.66; P 0.016), suggesting its relevance even equalizing for baseline clinical status.

There was no correlation between AH-SAI and either slope of the input–output curve or site of the lesion. Also, there was no correlation between site of the lesion and recovery at 6 months.

Involvement of cholinergic pathways was limited and there was no correlation between AH-SAI and either percentage of lesional voxels of medial pathway, lateral cholinergic pathways and lateral perforant pathway or percentage of lesional voxels in the 3 pathways considered together.

Paper 3: Immediate and late modulation of interhemispheric imbalance with bilateral transcranial direct current stimulation in acute stroke

Clinical outcome

Experiment 1. For each clinical measure (NIHSS, mRS, ARAT, NHPT, NIHSS, MAL (AOU), MAL (QOM)) we computed a repeated measure ANOVA with Time as within subject factor (2 levels: baseline, 1 week) and tDCS (two levels: real and sham) as between subject factors. tDCS had no effect on clinical outcome (tDCS and the tDCS by Time interaction: $P > 0.200$ consistently). We confirmed the expected and well-known time-related clinical improvement (factor Time $P < 0.001$ for all the measures considered).

Experiment 2. The repeated measure ANOVA with Time as within subject factor (3 levels: base, 1 week, 3 months) and tDCS (2 levels: real and sham) as between subject factor showed the absence of tDCS effect on clinical outcome (factor tDCS and tDCS by Time interaction: $P > 0.050$ consistently).

Cortical excitability

Experiment 2. The aim of the analysis was to check whether there was a change of brain excitability across hemispheres, time and groups.

The two groups (real tDCS vs. sham tDCS) had no different excitability at baseline (t0), as demonstrated by the lack of tDCS factor effect (Wald chi-square 1⁄4 0.352, df 1⁄4 1, P 1⁄4 0.553) and of

Hemisphere by tDCS interaction (Wald chi-square 1⁄4 066, df 1⁄4 1, P 1⁄4 0.797). The inter-hemispheric excitability imbalance, well-known in acute stroke, was confirmed in both groups by the presence of a main effect of Hemisphere (Wald chi-square 1⁄4 15.334, df 1⁄4 1, P < 0.001).

The further step was to compare the excitability of the two groups (real tDCS and Sham tDCS) along the time-course of the investigation computing a GEE model with Patient as “Subject” (or cluster) variable, MEP values as dependent variable and Hemisphere (AH and UH), Time (t0, t1, t2) and tDCS (Real and Sham) as predictors. The presence of a significant triple interaction Time by Hemisphere by tDCS (Wald chi-square 1⁄4 31.652, df 1⁄4 7, P < 0.001) showed that the inter-hemispheric balance of excitability is differently modulated along time accordingly with tDCS exposure (real tDCS and Sham tDCS).

In the context of the effect of tDCS on the inter-hemispheric balance, the Real_tDCS group, compared to the Sham_tDCS group, showed a reduction of LI when considering both t2 t1_norm (P 1⁄4 0.007) and t2 t0_norm (P 1⁄4 0.015).

In summary, the main effect of tDCS was the reduction of inter-hemispheric imbalance at 3 months of follow-up.

LTP/LTD-like changes promoted by iTBS

Experiment 2. We wanted to evaluate whether the addition of tDCS to a CIMT protocol can change the amount of LTP of the AH and of LTD of UH promoted by iTBS applied over the AH. We computed a GEE model with Patient as “Subject” (or cluster) variable, MEP values as

dependent variable and Hemisphere (AH and UH), tDCS (Real and Sham), Time (t0, t1, t2) and TBS (Pre-TBS and Post-TBS) as predictors.

The presence of a significant Hemisphere by Time by TBS by tDCS interaction (Wald chi-square 1⁄4 41.472, df 1⁄4 14, P < 0.001) suggests that the effect of TBS on each hemisphere differently changes along time in the two groups.

Once the GEE model with Hemisphere (AH and UH), Time (t0, t1, t2) and TBS (Pre-TBS and Post-TBS) as predictors was applied separately for each group, we found a significant Hemisphere by Time by TBS interaction only for the Real_tDCS group (Real_tDCS: Wald chi-square 1⁄4 28.870, df 1⁄4 7, P < 0.0001; Sham_tDCS: Wald chi- square 1⁄4 6.350, df 1⁄4 7, P 1⁄4 0.499). Therefore, in other words, the “Real tDCS” supports a different modulation of TBS effects across Hemispheres and Time. The visual inspection of the curves suggests that at t1 iTBS produced both greater increase of AH excitability and decrease of UH excitability in patients exposed to Real_tDCS.

Finally, the post-iTBS study of LI suggests that real tDCS decreases inter-hemispheric imbalances compared to Sham_tDCS. In other words, tDCS improves baseline inter-hemispheric balance and also TBS after-effects on the AH.

Paper 4: Inhibitory theta burst stimulation of affected hemisphere in chronic stroke: A proof of principle, sham-controlled study.

There were no adverse events. Age, time from the acute event, NIHSS, BI, lesion side, M/F ratio and residual hand function as evaluated by ARAT and JTT baseline scores were similar in the two studied groups.

ARAT

Repeated measures ANOVA showed a significant effect of TIME, but no significant TIME×GROUP interaction. Thus, physical therapy was effective regardless the rTMS intervention. Nevertheless, we explored the effect of TIME in more details by a within-group comparison examining changes from baseline in each group (real- and sham-cTBS) with paired t-tests. Both real- and sham-cTBS groups improved significantly at T1, T2 and T3. Patients undergoing real cTBS increased mean ARAT score above baseline by about 30% at T1 and by about 36% at T2, T3. The sham group increased about 27% at T1, about 30% at T2 and about 34% at T3.

JTT

Repeated measures ANOVA for JTT execution time showed a significant effect of TIME and a significant TIME × GROUP interaction. This simple measure of the time taken to perform the JTT might not

provide a good description of the data if patients cannot perform any of the tasks at baseline, but then succeed after treatment. To account for this, we also used a normalized score in which we express the performance of the affected side as a percentile of the performance of the unaffected side. A repeated measures ANOVA on these normalized scores showed a significant effect of TIME but no significant TIME × GROUP interaction. However, a within-group comparison (both for performance time and normalized scores) revealed that only the patients who underwent real cTBS improved at T1, T2 and T3 ($p = 0.01$, $p = 0.01$ and $p = 0.015$ respectively for performance time; $p=0.01$, $p=0.02$ and $p=0.03$ respectively for normalized scores), whereas no significant effect of TIME was found in the sham group. In patients undergoing real cTBS, mean JTT execution time was reduced compared to baseline by 116 s at T1, 147 s at T2 and 141 s at T3 whereas for the sham group the mean reduction in performance time was 51, 63 and 65s at the same time points; furthermore normalized scores increased compared to baseline by about 55% at T1, 70% at T2 and 60% at T3, whereas for the sham group the mean increase was between 15% and 22% at the same time points. Between groups post hoc analysis revealed no significant differences at each time point for both measuring methods.

Motor cortex excitability

At baseline paired t-test revealed a significant difference in MEP amplitude between UH and AH in both subgroups [(UH vs AH); real group: 606 ± 240 V vs 160 ± 177 V ($p = 0.01$): sham group: 421 ± 205

V vs 90 ± 75 V (p = 0.01)]. No significant difference at baseline was disclosed in motor thresholds and MEP amplitude for both hemispheres between the two studied groups. ANOVA revealed no significant effect of TIME nor significant TIME × GROUP interaction for both motor threshold and for MEP amplitude. No significant change in AMT was seen at different time points for both studied groups

Discussion

By means of this "*excursus*", it was demonstrated that:

1. Neuromodulation techniques, although in healthy subjects could lead to similar effects (same direction and similar effect size), differ in the underlying mechanisms of action, especially when observe them at cellular and intracellular level. It was indirectly demonstrated by studying the cohort of CS patients: the presence of a single mutation in a ubiquitous intracellular protein cascade involved in learning and plasticity influence the after-effects of two similar facilitatory rTMS protocols in a differential manner (paper 1).
2. RTMS-induced (and for deduction of all kinds of neuromodulation protocols) LTP-like after-effects are not always correlated with good learning processes and if excessive could be detrimental (paper 1)
3. By studying motor cortex excitability in the acute phases after a stroke, it is possibile to better characterize the patients in terms of their "natural" tendency to motor recovery (paper 2).
4. Increasing the excitability of the AH and reducing the excitability of the UH, besides the induced neurophysiological changes, doesn't lead to a clinical improvement in acute stroke patients in terms of motor recovery (paper 3).

5. Conversely, reducing the excitability of the AH in chronic stroke patients induce an amelioration in motor tasks in the paretic hand without any side effects (paper 4).

Hence, it could be firstly concluded that non-invasive brain stimulation techniques have a potentially central role in studying stroke patients and their propensity to motor recovery. Indeed, TMS permits a non-invasive, non-painful, feasible and safe evaluation of motor cortex excitability in stroke patients. Furthermore, neuromodulation, in the form of rTMS or tDCS, could be a useful and effective add-on treatment in the post-stroke phase with the ultimate aim to achieve an improvement of the degree of motor recovery.

If boosting the effects of physical therapy is achieved, it could be possible to reduce the social costs of the illness, by reducing the global time of rehabilitation (as well as in inpatients or outpatients clinics modality) and increase the quality of life after stroke in the survived cohort.

Ras signalling plays a key-role on the structural and functional synaptic connectivity within cerebral cortex and is strictly connected to synaptic LTP [97], motor learning and memory [98]. It should be noted that the differential influence of HRAS mutation on the after-effects of two similar (i.e. facilitatory) rTMS techniques suggests that non-invasive brain stimulation techniques, as far as inducing in healthy subjects the same gross effects, could activate different intracortical pathways inducing LTP. Indeed, some differences in the neuromodulatory mechanisms of PAS and iTBS were already demonstrated in

Parkinson's Disease (PD) patients in which L-dopa was able to restore abnormal plasticity induced by PAS [99-101] but not the abnormal response to iTBS [102]. Moreover this hypothesis fits well with the experimental evidence that different stimulation protocols could activate different pathways inducing LTP and with the evidence of a differential activation of different cortical circuits [103] by means of different NIBS protocols [104]. If this was the case, this concept could be generalized to all the neuromodulation protocols, in the sense that not all the stroke patients could benefit from the same protocol, depending on their genetic background and specific condition. Furthermore, HRAS mutation in CS is associated to a paradoxical increase in LTP associated to decreased motor function in animal models: the "in vivo" replication of these findings by means of NIBS protocols could suggest that the artificial increase in LTP could be detrimental and not beneficial (paper 1). Furthermore it was also shown that downregulation of Ras-dependent cascade could enhance motor recovery in experimental models of stroke [105].

Altogether these considerations suggest that the choice of a NIBS protocol as a technique boosting motor recovery after stroke, and, in general, as a potential add-on treatment in neurological illnesses, could be the first crucial step to achieve more effective results.

Moreover, we observed a suppression of SAI in acute stroke patients and, remarkably, that AH-SAI level was inversely correlated with recovery at 6 months. Since SAI is probably mediated by the α5-subunit of GABA receptor [106], and since pharmacological antagonization of

α5-subunit activity was showh to promote functional recovery after stroke [107], we hypothesized that SAI suppression, and its correlation with motor improvement, might be related to a reduction of activity related to this subunit. These findings are very interesting and could suggest a tailored rehabilitation and neuromodulation approach for each stroke patients depending on the level of SAI measured in the acute phase. In other words, patients with less reduced SAI could benefit of larger and “aggressive” rehabilitation scheme while patients with a more reduced SAI (theoretically more prone to a good recovery) could benefit of more intensive, earlier and shorter rehabilitation schemes.

Nevertheless, when a contemporary inhibition of the excitability of healthy hemisphere and a facilitation of the affected hemisphere were induced by means of bilateral tDCS (delivered for 40 minutes daily for 5 consecutive days) during the acute phase after a stroke (paper 3), a significantly reduction of inter-hemispheric imbalance between AH and UH was found without any significant change in hand motor function. In other words alleviating the inter-hemispheric imbalance didn’t translate into a better motor function for the paretic hand. Contemporary we demonstrated that NIBS application in acute stroke could be considered a safe procedure and that bilateral tDCS was able to increase LTP-like effects in AH at 3 months. In line with present results, Rossi and co-workers showed that five-daily sessions of anodal tDCS applied to the motor cortex of the AH in acute stroke patients appear to be safe, but do not improve clinical outcomes [108]. Considering the relative small cohort of recruited patients in both studies, it could also be conceivable

that clinical negative results were due to the intra-cohort variability related to lesion size, affected hemisphere, lesion site (cortical vs subcortical) as well as age, risk factors and so on and our study was underpowered. However, these data seem to suggest two major considerations:

1. In acute stroke, the level of plasticity could be significantly dysfunctional or, if considered compensatory, it already reaches a ceiling effect that does not allow five days of tDCS to produce additional evident clinical benefits.
2. Since the neurophysiological rebalancing of inter-hemispheric motor cortex excitability didn't translate in clinical improvement, it could be suggested that the competition model is not the adequate rationale to follow (at least in the acute phase) for promoting motor recovery after a stroke and the application of other strategies should be investigated.

In line with this last consideration, we could hypothesize that the increase of UH excitability as well as the reduction of AH excitability could be considered a compensatory and not purely detrimental phenomenon in both acute and chronic phases after a stroke.

So, the relative failure of the previous study seems to indicate also the relative failure of the inter-hemispheric competition model.

In this line, we tried another small trial of NIBS application in stroke patients (in this case, chronic), in which we applied the "alternative" rationale of the compensatory model [88].

Following this last consideration in a different study (paper 4) we tried to follow the “compensatory/vicariation” rationale and applied an inhibitory rTMS protocol (i.e. cTBS) over AH in a cohort of chronic paretic stroke patients. We found no deleterious effects of cTBS both on safety matters and on recovery measures (i.e. a worsening in motor function), as might have been expected if competition model was working: all the patients showed some improvement in motor function of the paretic hand. Specifically, all the patients (in both real and sham groups) achieved sustainable improvements in the ARAT, NHPT and JTT, illustrating the success of the physical therapy. However, only patients who underwent real cTBS had a significant improvement in JTT. Contemporary no change in bilateral motor cortex excitability were observed. Unfortunately we didn’t study the acute neurophysiological after-effects of a single session of cTBS on AH, to disclose the presence of a different response when compared with healthy subjects and to have more mechanistical data. So, we hypothesized that inducing LTD-like changes in AH might enhance and/or accelerate the learning process and the effects of rehabilitation: this mechanism could fall into the concept of metaplasticity, the lower the neural excitability the higher the probability of consolidated motor learning [24]. However, it could be possible that cTBS had induced a reduction of interference from the stroke hemisphere towards the healthy hemisphere disinhibiting UH ipsilateral corticospinal control over paretic hand, as suggested by Bradnam and colleagues [109].

Whatever the explanation, with an adequate caution in considering these data definitive, this research trajectory seems suggesting that the inter-hemispheric competition model (that guided the majority of the studies about enhancing motor recovery in stroke patients) is not the optimal one. Moreover, it seems evident that the "compensatory/vicariation" model has good potentialities when applied to the theories about motor recovery mechanisms.

It should be considered that an important factor that could influence the choice of NIBS technique boosting the effects of physical therapy and the theoretical model more suitable to obtain a better motor recovery is represented by post-stroke phase in which we are acting (acute vs chronic).

Further, larger cohorts' studies are warranted to better define this scenario.

Conclusions

Motor deficit after a brain lesion, nowadays, represents the most frequent cause for disability in the world. Physical therapy, even was proven to be effective in recovery, is not sufficient to reach a satisfactorily level of quality of life after a stroke. For these reasons, neuromodulation was advocated as a potentially useful tool to boost the after-effects of physiotherapy: unfortunately the data from studies in which neuromodulation was paired to physical therapy didn't show yet conclusive results.

Here, when all the papers included in this compendium were evaluated at a glance, it jumps to attention that:

1. Similar neuromodulation protocols are characterized by concrete subtle differences in the underlying mechanisms of action that could affect the choice in the application of one protocol instead of another one; patients that seem not to beneficiate from a neuromodulation protocol could beneficiate from another one (paper 1).
2. Short latency afferent inhibition (studied in the early phase after a stroke) could differentiate between patients prone to better recovery and those who not, directing the single patients towards different more convenient and tailored therapeutic paths (paper 2).

3. Particular attention should be paid on the functional role of the excitability and plasticity changes that occur after a stroke in each patient: it could be that, in general, the inter-hemispheric competition model was not always the optimal option as demonstrated in the acute stroke patients in paper 3.
4. Changing the strategy of neuromodulation application, from inter-hemisperic competition/rivalry model to compensatory/vicariation model could effectively enhance the after-effects of physical therapy (paper 4).

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Toledo, 18th may 2017

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